

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 6 :</b> <b>C12N 15/86, 15/87, 5/10, A61K 35/76, 48/00</b>		<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 96/39530</b> <b>(43) International Publication Date:</b> 12 December 1996 (12.12.96)
<b>(21) International Application Number:</b> <b>PCT/US96/10245</b>		Robindale Apartment F5, 1925 Lawrence Road, Havertown, PA 19083 (US).	
<b>(22) International Filing Date:</b> 4 June 1996 (04.06.96)		<b>(74) Agents:</b> BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).	
<b>(30) Priority Data:</b> 08/462,014 5 June 1995 (05.06.95) US 08/549,489 27 October 1995 (27.10.95) US		<b>(81) Designated States:</b> AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
<b>(60) Parent Applications or Grants</b> <b>(63) Related by Continuation</b> US 08/462,014 (CIP) Filed on 5 June 1995 (05.06.95) US 08/549,489 (CIP) Filed on 27 October 1995 (27.10.95)		<b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>	
<b>(71) Applicant (for all designated States except US):</b> THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; 133 South 36th Street, Philadelphia, PA 19104-3246 (US).			
<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> WILSON, James, M. [US/US]; 1350 N. Avignon Drive, Gladwyne, PA 19035 (US). FISHER, Krishna, J. [US/US]; 4006 Pine Street, Philadelphia, PA 19104 (US). GAO, Guang-Ping [CN/US];			
<b>(54) Title:</b> RECOMBINANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS, CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF			
<b>(57) Abstract</b>  An adenovirus E1/E4 expressing packaging cell line is provided, which permits the generation of recombinant adenoviruses deleted in both gene regions. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell is provided by infecting a target cell with a recombinant AAV comprising a selected transgene under the control of regulatory sequences. The infected cell is contacted with an agent which facilitates the conversion of single stranded recombinant virus to its double stranded form.			

BEST AVAILABLE COPY

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

RECOMBINANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS,  
CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF

This invention was supported by the National  
Institute of Health Grant Nos. HD32649-01, DK47757 and  
5 DK49136. The United States government has rights in this  
invention.

Field of the Invention

The present invention relates generally to the field  
of somatic gene therapy, and specifically to methods and  
10 compositions useful in the treatment of genetic  
disorders.

Background of the Invention

Adenoviruses are eukaryotic DNA viruses that can be  
modified to efficiently deliver a therapeutic or reporter  
15 transgene to a variety of cell types [see, e.g., M. S.  
Horwitz et al, "Adenoviridae and Their Replication",  
Virology, second edition, pp. 1712, ed. B. N. Fields et  
al, Raven Press Ltd., New York (1990)]. Recombinant  
adenoviruses (rAds) are capable of providing extremely  
20 high levels of transgene delivery to virtually all cell  
types, regardless of the mitotic state. The efficacy of  
this system in delivering a therapeutic transgene *in vivo*  
that complements a genetic imbalance has been  
demonstrated in animal models of various disorders [K. F.  
25 Kozarsky et al, Somatic Cell Mol. Genet., 19:449-458  
(1993) ("Kozarsky et al I"); K. F. Kozarsky et al, J.  
Biol. Chem., 269:13695-13702 (1994) ("Kozarsky et al II")  
and others]. The use of recombinant adenoviruses in the  
transduction of genes into hepatocytes *in vivo* has  
30 previously been demonstrated in rodents and rabbits [see,  
e.g., Kozarsky II, cited above, and S. Ishibashi et al,  
J. Clin. Invest., 92:883-893 (1993)].

The first-generation recombinant, replication-  
deficient adenoviruses which have been developed for gene  
35 therapy contain deletions of the entire E1a and part of  
the E1b regions. This replication-defective virus is

grown on an adenovirus-transformed, complementation human embryonic kidney cell line containing a functional adenovirus E1a gene which provides a transacting E1a protein, the 293 cell [ATCC CRL1573]. E1-deleted viruses 5 are capable of replicating and producing infectious virus in the 293 cells, which provide E1a and E1b region gene products *in-trans*. The resulting virus is capable of infecting many cell types and can express the introduced gene (providing it carries its own promoter), but cannot 10 replicate in a cell that does not carry the E1 region DNA unless the cell is infected at a very high multiplicity of infection.

Adeno-associated virus (AAV) is an integrating human DNA parvovirus which has been proposed for use as a gene 15 delivery vehicle for somatic gene therapy [B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168 (1990)]. This small non-enveloped virus contains a 4.6 kb single stranded (ss) DNA genome that encodes sets of regulatory and capsid genes called 20 rep and cap. Rep polypeptides (rep78, rep68, rep62 and rep40) are involved in replication, rescue and integration of the AAV genome. The cap proteins (VP1, VP2 and VP3) form the virion capsid. Flanking the rep and cap open reading frames at the 5' and 3' ends are 145 25 bp inverted terminal repeats (ITRs), the first 125 bp of which are capable of forming Y- or T-shaped duplex structures.

Recombinant forms of AAV (rAAV) have been developed 30 as vectors by replacing all viral open reading frames with a therapeutic minigene, while retaining the necessary cis elements contained in the ITRs. [See, e.g., US Patent Nos. 4,797,368; 5,153,414; 5,139,941; 5,252,479; and 5,354,678; and International Publication Nos. WO 91/18088 published November 28, 1991; WO 93/24641 35 published December 9, 1993 and WO94/13788 published June

23, 1994]. However, progress towards establishing AAV as a transducing vehicle for gene therapy has been slow for a variety of reasons. For example, the integrated provirus preferentially targets specific sites in 5 chromosome 19. Additionally, difficulties surround large-scale production of replication defective recombinants. The cells employed to produce rAAV must also be infected with adenovirus or herpesvirus to provide the necessary helper functions, thereby producing problems in purifying 10 recombinant AAV (rAAV) from contaminating virus in culture. Practical experience with purified recombinant AAV as a gene therapy vector has been disappointing, because the more purified the AAV is from co-infection 15 with its helper virus in culture, the lower the gene transduction efficiencies that the rAAV displays.

There remains a need in the art for additional recombinant adenoviruses and rAAV, therapeutic compositions and methods which enable effective use of these recombinant viruses in the treatment of disorders 20 and diseases by gene therapy.

Summary of the Invention

In one aspect of this invention, a packaging cell line is provided which expresses adenovirus genes E1a, 25 E1b and E4, or functional fragments thereof, e.g., the E4 open reading frame (ORF) 6.

In another aspect, the invention provides a rAd comprising the DNA of at least a portion of the genome of an adenovirus having functional deletions of the E1 and E4 gene regions; a suitable gene operatively linked to 30 regulatory sequences directing its expression, and an adenovirus capsid, the rAd capable of infecting a mammalian cell and expressing the gene product in the cell *in vivo* or *in vitro*. The invention also provides a mammalian cell infected with the rAd described above.

In still another aspect, the invention provides a rAd shuttle vector comprising the DNA of at least a portion of the genome of an adenovirus having functional deletions of the E1 and E4 gene regions.

5 In a further aspect, the invention provides a method for producing the above-described recombinant Ad and a method for delivering a selected gene into a mammalian cell using the recombinant Ad described above.

In another aspect, the invention provides a method  
10 for enhancing the efficiency of transduction of a recombinant AAV into a target cell. The method operates, in brief, by infecting a target cell with a ss recombinant adeno-associated virus (rAAV) which comprises a transgene operatively linked to regulatory sequences  
15 directing its expression, and contacting the infected cells with an agent which facilitates the conversion of ss rAAV to its double stranded (ds) form. Conversion of ss rAAV to ds rAAV occurs in the target cell, resulting in enhanced transduction of the rAAV into the target  
20 cell. The agent may be a helper virus which carries a selected gene or functional fragment thereof encoding a polypeptide capable of enhancing the conversion of the ss rAAV to ds rAAV and which is co-infected into the same target cell. The agent may also be a drug or chemical  
25 composition which accomplishes the same function and is applied to the infected target cell. This method can operate both in an ex vivo setting and in vivo.

In yet another aspect, the invention provides a novel recombinant AAV, which contains both the transgene  
30 intended for use in treating a genetic disease or disorder and at least one additional gene operatively linked to inducible or constitutive regulatory sequences. The additional gene(s) encodes a polypeptide capable of facilitating, alone or in concert with other additional  
35 genes, the conversion of ss rAAV to its ds form upon

expression. In a preferred embodiment, the additional gene is adenovirus E4 or a functional fragment thereof. Also disclosed is a method for enhancing the efficiency of transduction of the novel rAAV into a target cell.

5 The novel rAAV and methods of this invention are also useful in pharmaceutical compositions for use in ex vivo and in vivo gene therapy treatment protocols for treating inherited diseases, cancer, and other genetic dysfunctions.

10 Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

15 Fig. 1 is a schematic drawing of an exemplary plasmid pMMTVE4ORF6 [SEQ ID NO: 1] or pMTE4ORF6, which contains an MMTV or sheep MT promoter, respectively, in control of a human E4 ORF 6 gene sequence, a growth hormone gene terminator sequence (GH), an SV40 ori, pBR322-based plasmid sequences including a neo<sup>R</sup> gene, an 20 SV40 polyA site and an amp<sup>R</sup> gene.

25 Fig. 2 is a schematic map of rAd H5.001CBLacZ [SEQ ID NO: 3] with indicated restriction endonuclease enzyme sites. The striated bar represents the CBLacZ minigene; the black bar represents Ad5 viral backbone, the crosshatched bar represents Ad E4 deletion.

Fig. 3 plots LacZ forming units (LFU)/ml vs time (hours) for E4 complementing cell lines infected with H5.001CBLacZ.

30 Fig. 4A is a graph of the induction, ORF6 expression and viral production in 293-27-18 packaging cells plotting yield at 24 hours post-infection (pi) in LFU/ml and ORF6 protein (abs.mm) vs. concentration of the inducer, dexamethasone ( $\mu$ M). Abs.mm is the intensity of the size of the protein band on a Western blot and 35 reflects absorbance and protein size in mm<sup>2</sup>. The square

is yield at 24 hours pi. The diamond is ORF6 protein detected at 24 hours pi.

Fig. 4B is a similar graph to that of Fig. 4A, except that the packaging cells are 293-10-3 cells. The 5 symbols are as described for Fig. 4A.

Fig. 5A is a bar graph plotting  $\beta$ -galactosidase enzyme activity in lysates from infected HeLa cells. The horizontal axis indicates the adenoviruses infected into the HeLa cells, with the symbol "+" indicating the 10 addition of the adenovirus to the rAAV, AV.CMVLacZ. The vertical axis indicates intracellular  $\beta$ -galactosidase specific activity (mUnits/mg protein) using ONPG. Below each bar, the fold-induction in specific activity relative to cells that received the AV.CMVLacZ vector 15 alone is given.

Fig. 5B is a bar graph plotting Ad multiplicity of infection (MOI) in HeLa cells of wild-type Ad5 or the E2 mutant dl802, the cells co-infected with rAAV vs. 20 intracellular  $\beta$ -galactosidase specific activity. See Example 11.

Fig. 6A is a graph in which  $\beta$ -galactosidase specific activity and counts per minute (CPM) are plotted along the vertical axis and adenovirus MOI's are on the horizontal axis for HeLa cells infected with wtAd5 and 25 rAAV according to Example 12. Data obtained from low MOI (1, 5, and 10) infections are shown.

Fig. 6B is a graph similar to that of Fig. 6A except that the cells were infected with Ad mutant dl802.

Fig. 7A illustrates a model for leading strand 30 synthesis of a complementary AAV strand in the presence of Rep (+Rep) or absence of Rep (-Rep). Rep expresses a terminal resolution activity that can convert a duplex structure with closed-ends to an open-ended duplex. In the absence of Rep, terminal resolution is impaired 35 leaving the covalently closed, hairpin structures intact.

Under these conditions, hairpins are expected to be found leftward and rightward, since both strands of a rescued ds AAV genome are packaged into virions.

Fig. 7B is a schematic of linear AV.CMVLacZ with 5 labeled domains including the AAV ITRs, CMV immediate early enhancer/promoter (CMV), SV40 splice donor-splice acceptor (SD/SA), *E. coli*  $\beta$ -galactosidase cDNA (*LacZ*), and SV40 polyA signal (pA). Two NotI sites located at bp positions 1035 and 4509 are indicated.

Fig. 7C illustrates a closed end and an open end 10 fragment of rAV.CMVLacZ.

Figs. 7D, 7E and 7F indicate the mixture of 15 open-ended and covalently closed duplex fragments generated by NotI digestion of ss AV.CMVLacZ at position 4509 in the absence of terminal resolution. The NotI 4509 digestion provides a convenient means of releasing a 361 bp fragment that contains the right ITR in the context of a hybridization target (i.e. SV40 pA). In the presence of terminal resolution, only the open-ended 361 20 bp fragment would be expected to be generated (Fig. 7D) by such digestion.

Fig. 8A is a bar graph plotting  $\beta$ -galactosidase 25 specific activity (mUnits/mg protein) vs. increasing concentration of zinc ( $\mu$ M) inducer for cell line 293 (MT-ORF6) transduced with AVCMVLacZ (first row below each bar). Also provided is the fold-induction relative to 293 cells (second row below each bar), and the fold-induction relative to 293(ORF6) cells maintained in the absence of zinc (third row).

Fig. 8B is a bar graph plotting CPM of duplex 30 monomer replicative form (RFm) of rAAV vs. the concentration of zinc ( $\mu$ M) used for induction and the fold-induction relative to 293(ORF6) cells maintained in 0 mM zinc below each bar.

Fig. 8C is a graphical comparison of the induction profiles that describe AV.CMVLacZ transduction efficiency. Specific activity data from Fig. 8A and CPM data of AV.CMVLacZ RFm from Fig. 8B are plotted along the vertical axis, and concentration of zinc sulfate used during the experiment is shown along the horizontal axis.

Fig. 9 is a bar graph plotting specific activity (milliunits  $\beta$ -galactosidase/mg protein) vs the concentration of zinc used for induction (first row under the horizontal axis), the fold-induction relative to HeLa cells (second row), and the fold-induction relative to HeLa(Mt-ORF6) cells maintained in the absence of zinc (third row), for the HeLa(MT-ORF6) cells transduced at an MOI of 1,000 AV.CMVLacZ virus particles/cell in the absence of zinc sulfate inducer or in the presence of 50, 100, 150, 200 or 250  $\mu$ M zinc sulfate inducer.

Fig. 10 is a schematic of the plasmid pAV.CMVLacZ [SEQ ID NO: 4].

Fig. 11 illustrates plasmid pAV.CMVALP.GRE-ORF6 [SEQ ID NO: 5].

#### Detailed Description of the Invention

The present invention provides packaging cell lines, which enable the production of recombinant adenoviruses (rAd) functionally deleted in both the E1 and E4 genes. These rAd and methods which enable the therapeutic treatment of disorders with such rAds are disclosed. Novel "second generation" recombinant adeno-associated virus (rAAV) and methods for enhancing the transduction efficiency of rAAV containing a transgene for expression in a somatic gene therapy protocol are also provided. The methods and compositions of this invention are useful in *ex vivo* applications of gene therapy, such as in the transduction of bone marrow cells with desirable hematopoietic stem cell progenitor genes prior to bone marrow transplantation. The embodiments of the invention

are also useful in pharmaceutical compositions for direct in vivo treatment of patients by gene therapy vectors, including the transduction of desirable genes in patients with genetic disorders, such as cystic fibrosis.

5       *I. Packaging Cell Lines*

To increase the transgene capacity and decrease immune response of rAds, as many viral genes as possible should be deleted to inactivate the adenovirus. However, it is crucial to generate complementing cell lines for 10 construction and propagation of such deleted Ad. The method and compositions of the present invention overcome several problems previously identified in the gene therapy for first generation E1 deleted adenoviruses and display advantages in administration particularly to 15 muscle tissue.

Early region 4 (E4) of Ad serotype 5 consists of 7 ORFs believed to be involved in viral DNA replication, host cell shut-off, and late mRNA accumulation. To generate rAd deleted in E4, the function of the E4 region 20 must be supplied to the rAd by a helper virus or packaging cell line. However, useful packaging cell lines have not been available previously because normally the continuous expression of functioning Ad E1 and functional E4 in a single cell line are toxic to the 25 cell. Such cells are therefore not useful for the growth and replication of rAds. Further, the DNA encoding the functional Ad E1 and Ad E4 genes, when present in a packaging cell line, can increase the chances of recombination with a rAd virus to cause the virus to 30 revert to a wildtype Ad virus.

The present invention avoids these problems by providing a packaging cell line which contains the Ad5 E1 gene and only the ORF 6 of the Ad5 E4 gene. ORF6 of E4 alone can provide the requirements for E4 in the viral 35 life cycle.

According to this invention, the ORF6 is preferably under the transcriptional control of an inducible promoter. The mouse mammary tumor virus (MMTV) promoter, inducible by a glucocorticoid, particularly, 5 dexamethasone, is presently preferred. The DNA sequence of the MMTV promoter spans nucleotides 1-1506 of SEQ ID NO: 1. Another inducible promoter is the sheep metallothioneine (MT) promoter, inducible by zinc [M. G. Peterson et al, *Eur. J. Biochem.*, 174:417-424 (1988)]. 10 However, the zinc sulfate inducer of the MT promoter can itself be toxic to the cells. Other inducible promoters, such as those identified in International patent application WO95/13392, published May 18, 1995, and incorporated by reference herein may also be used in the 15 production of packaging cell lines according to this invention. Constitutive promoters, such as the constitutive Ad5 E4 region promoter, LTR, may be employed in control of the expression of ORF6.

The packaging cell line of the invention which 20 utilizes an inducible promoter permits one to control the development of toxicity by regulating the expression of the E4 ORF6 gene. After the desired shuttle vector containing the Ad sequences is transfected into the cell line, expression of the E4 ORF6 can be induced by the 25 appropriate inducer. The packaging cell is thus able to provide both Ad E1 and Ad E4 ORF6 gene products to the rAd for a sufficient period to allow productive infection and recovery of the rAd, before the cell becomes toxic. At present, the time period before the cell experiences 30 toxicity is about 10 days.

In its most preferred form, the packaging cell line is a human embryonic kidney (HEK) 293 E1 expressing cell line into which is introduced the E4 ORF 6 sequence under the control of the inducible promoter. It should be 35 understood by one of skill in the art that another parent

cell line may be selected for the generation of a novel cell line expressing the E1a, E1b, and E4 ORF6 genes of a selected adenovirus serotype. Among such parent cell lines may be included HeLa [CCL 2], A549 [CCL 185], KB [CCL 17], Detroit [e.g., Detroit 510, CCL 72] and WI-38 [ATCC CCL 75] cells. These cell lines are all available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA. Other suitable parent cell lines may be obtained from other sources. If such parent cell lines were selected for modification, the cell line would need to be further supplied with the E1a and E1b gene functions, e.g., such as by transfection with a plasmid containing these genes or functional fragments thereof under a suitable promoter, as well as with the ORF6 gene as described herein.

Example 1 teaches construction of packaging cell lines containing only the ORF 6 of Ad5 E4 region or, for functional comparisons, the entire E4 region. Briefly described, the entire E4 region and an ORF6 sequence of Ad 5 E4 gene were obtained by known techniques [see, e.g., Sambrook et al., "Molecular Cloning. A Laboratory Manual.", 2d edit., Cold Spring Harbor Laboratory, New York (1989) and references cited therein]. To isolate the ORF6 region, the anchored PCR technique was used to amplify the ORF6 sequence from its initiation codon to its termination codon. Primers selected from the published sequence of ORF6 were used to amplify the ORF sequence and insert restriction sites onto the end of the sequence. The E4 ORF6 sequence itself is reproduced as nucleotides 1523 through 2408 of SEQ ID NO: 1. The entire E4 gene sequence is published in the Genbank sequence of Ad5 [Genbank Accession No. M73260].

A minigene was constructed that placed the ORF6 sequence under the control of a selected promoter. By "minigene" as used here is meant the combination of the

desired sequence to be expressed (in this particular instance, the ORF6 sequence) and the other regulatory elements necessary to transcribe the desired sequence and express the gene product in a cell containing that 5 minigene. The ORF6 sequence gene is operatively linked to regulatory components in a manner which permits its transcription. Such components include conventional regulatory elements, such as a promoter to drive ORF6 expression. One inducible promoter was the Zn<sup>+2</sup> 10 inducible MT promoter; the other was the dexamethasone-inducible MMTV promoter of SEQ ID NO: 1.

The minigene also contains nucleic acid sequences heterologous to the ORF6 viral sequence, including sequences providing signals required for efficient 15 polyadenylation of the transcript (poly-A or pA). A common poly-A sequence which is employed in this invention is that derived from the growth hormone (GH) gene terminator sequence (nuc. 2409-3654 of SEQ ID NO: 1). The poly-A sequence generally is inserted in the 20 minigene following the ORF6 sequence. The polyA sequence employed in the MMTV-ORF6 minigene described in Example 1 [SEQ ID NO: 1] is supplied by the GH gene terminator and an SV40 origin of replication (ori). A similar minigene differing in promoter sequence, polyA sequence and/or 25 SV40 ori can also be designed by one of skill in the art to transfer the E4 ORF6 sequence to a shuttle plasmid. Selection of these and other common vector elements are conventional [see, e.g., Sambrook et al, cited above, and references cited therein] and many such sequences are 30 available from commercial and industrial sources as well as from Genbank.

The ORF6-containing minigene was subcloned into a pBR322-based shuttle plasmid that contained a neomycin resistance gene, resulting in the shuttle vector of Fig. 35 1. Any of the many known bacterial shuttle vectors may

be employed to carry the minigene, providing that the vector contains a reporter gene or selectable marker of which many, e.g., neo, amp or purimycin, are known in the art. It is expected that one of skill in the art can 5 develop other suitable shuttle vectors using other plasmid components which are similarly capable of transferring the ORF6 minigene into the chromosome of a cell transfected with the plasmid.

As further described in Example 1, other shuttle 10 vectors were designed for comparative purposes, which contain the complete or substantially complete Ad5 E4 region under the control of the constitutive retroviral MLV LTR sequence in the presence or absence of the endogenous E4 promoter. The shuttle plasmid carrying the 15 ORF6 minigene (or the entire E4 region) was introduced into HEK 293 cells which express the Ad E1 gene products. Complementing cell lines were generated that express these Ad E4 or ORF6 genes from either their endogenous promoters or heterologous inducible promoters. These 20 cell lines are further characterized by their genetic constitution, E4 protein synthesis, recombinant AAV helper function, relative plaque efficiency of H5d11004 virus, and growth kinetics of recombinant E1/E4 deleted adenovirus. These characteristics of exemplary E1/E4 25 expressing packaging cell lines are discussed in detail in the following examples.

## II. Recombinant Adenovirus

The E1/E4 expressing cell line is useful in 30 constructing E1/E4 deleted rAds which can deliver a suitable gene to mammalian cells and tissues. These rAd are functionally deleted in at least the E1a, E1b and E4 Ad gene regions. By the term "functionally deleted" is meant that a sufficient amount of the gene region is removed or otherwise damaged, e.g., by mutation or 35 modification, so that the gene region is no longer

capable of producing the products of gene expression. If desired, the entire gene region may be removed. In *in vivo* experiments with the rAd grown in the packaging cell lines, the E1/E4 deleted rAd demonstrated utility  
5 particularly in transferring a transgene to a muscle cell.

---

The adenovirus sequences used in the construction of the shuttle vectors, helper viruses, if needed, and rAd, and other components and sequences employed in the 10 construction of the vectors and viruses described herein may be readily obtained from commercial or academic sources based on previously published and described sequences. Viral materials may also be obtained from an individual patient. The viral sequences and vector 15 components may be generated by resort to the teachings and references contained herein, coupled with standard recombinant molecular cloning techniques known and practiced by those skilled in the art. Modifications of existing nucleic acid sequences forming the vectors, 20 including sequence deletions, insertions, and other mutations taught by this specification may be generated using standard techniques. Similarly, the methods employed for the selection of viral sequences useful in a vector, the cloning and construction of the "minigene" 25 and its insertion into a desired viral shuttle vector and the production of a recombinant infectious virus are within the skill in the art given the teachings provided herein.

**A. Construction of the Transgene**

30 A "minigene" in this context is defined as above, except that the components of this minigene are designed to express the gene product *ex vivo* or *in vivo*. Such components include conventional regulatory elements necessary to drive expression of the transgene in a cell 35 transfected with the rAd. For this minigene, a selected

promoter is operatively linked to the transgene and located, with other regulatory elements, within the selected viral sequences of the recombinant vector. Selection of the promoter is a routine matter and is not a limitation of this invention. Useful promoters may be constitutive promoters or regulated (inducible) promoters, which will enable control of the amount of the transgene to be expressed. For example, a desirable promoter is that of the cytomegalovirus (CMV) immediate early promoter/enhancer [see, e.g., Boshart et al, Cell, 41:521-530 (1985)]. Another desirable promoter includes the Rous sarcoma virus LTR promoter/enhancer. Still another promoter/enhancer sequence is the chicken cytoplasmic  $\beta$ -actin (CB) promoter [T. A. Kost et al, Nucl. Acids Res., 11(23):8287 (1983)]. Other suitable promoters may be selected by one of skill in the art.

The minigene may also desirably contain nucleic acid sequences heterologous to the viral vector sequences including poly-A sequences and introns with functional splice donor and acceptor sites, as described above. The poly-A sequence generally is inserted in the minigene following the transgene sequences and before the 3' adenovirus sequences. A minigene of the present invention may also contain an intron, desirably located between the promoter/enhancer sequence and the transgene. Selection of these and other common vector elements are conventional as described above and many such sequences are available from commercial and industrial sources as well as from Genbank.

As above stated, the minigene is located in the site of any selected deletion in the rAd. In the E1/E4 deleted rAd H5.001CBLacZ, the transgene is located in the deleted E1 gene region. However, the transgene may be located elsewhere in the adenovirus sequence, as desired.

B. Production of Recombinant Adenovirus

Adenovirus sequences useful in this invention may include the DNA sequences of a number of adenovirus types, which are available from Genbank, including type 5 Ad5 [Genbank Accession No. M73260]. The adenovirus sequences may be obtained from any known adenovirus serotype, such as serotypes 2, 3, 4, 7, 12 and 40, and further including any of the presently identified 41 human types [see, e.g., Horwitz, cited above]. Similarly adenoviruses known to infect other animals may also be employed in the vector constructs of this invention. The selection of the adenovirus type is not anticipated to limit the following invention. A variety of adenovirus strains are available from the American Type Culture Collection, Rockville, Maryland, or available by request from a variety of commercial and institutional sources. In the following exemplary embodiment an adenovirus, type 5 (Ad5) is used for convenience.

However, it is desirable to obtain a variety of adenovirus shuttle vectors based on different human adenovirus serotypes. It is anticipated that a library of such plasmids and the resulting rAds would be useful in a therapeutic regimen to evade cellular, and possibly humoral, immunity, and lengthen the duration of transgene expression, as well as improve the success of repeat therapeutic treatments. Additionally the use of various serotypes is believed to produce rAd with different tissue targeting specificities. Additionally, the absence of adenoviral genes E1 and E4 in the rAd of this invention should reduce or eliminate adverse CTL responses which normally cause destruction of rAds deleted of only the E1 gene.

rAds of this invention are recombinant, defective adenoviruses (i.e., E1 deleted) which are also deleted completely or functionally of the E4 gene region.

Functional deletions of E4 gene regions may be assessed by assays of Examples 2 and 3, among other assays. rAds useful in this invention may optionally bear other mutations, e.g., temperature sensitive (ts) mutations in the E2a gene region, and deletions in the E3 gene regions.

An adenovirus of this invention contains a functional deletion of the adenoviral early immediate early gene E1a (which spans mu 1.3 to 4.5) and delayed early gene E1b (which spans mu 4.6 to 11.2). Similarly the adenovirus has a functional deletion of the whole E4 region (which spans mu 92 to 97.2), or of at least ORF6 of the E4 region. Gene regions which may be optionally deleted in the E1/E4 deleted rAd of this invention include all or a portion of the adenovirus delayed early gene E3 (which spans mu 76.6 to 86.2). The function of E3 is irrelevant to the function and production of the rAd.

The rAd of this invention may also have a mutation which results in reduced expression of adenoviral protein and/or reduced viral replication. For example, a ts mutation may be introduced into the adenovirus delayed early gene E2a (which spans mu 67.9 to 61.5). Among such mutations include the incorporation of the missense ts mutation in the (DBP)E2a region found in the Ad5 H5ts125 strain [P. Vander Vliet et al, J. Virol., 15:348-354 (1975)] at 62.5 mu. A single amino acid substitution (62.5 mu) at the carboxy end of the 72 kd protein produced from the E2a gene in this strain produces a protein product which is a ss DNA binding protein and is involved in the replication of adenoviral genomic DNA. At permissive temperatures (approximately 32°C) the ts strain is capable of full life cycle growth on HeLa cells, while at non-permissive temperatures (approximately 38°C) no replication of adenoviral DNA is

seen. In addition, at non-permissive temperatures, decreased immunoreactive 72 kd protein is seen in HeLa cells. See, e.g., J. F. Engelhardt et al, Hum. Gene Ther., 5:1217-1229 (1994); J. F. Engelhardt et al, Proc. Natl. Acad. Sci., USA, 91:6196-6200 (1994) and International patent application WO95/13392, published May 18, 1995, incorporated by reference herein.

However, it should be understood that other deletions in the adenovirus genome as previously described in the art or otherwise may also occur in the rAd of this invention. One minimal type of rAd can contain adenovirus genomic sequences from which all viral genes are deleted. More specifically, the adenovirus sequences may be only the *cis*-acting 5' and 3' inverted terminal repeat (ITR) sequences of an adenovirus (which function as *oris*) and the native 5' packaging/enhancer domain, that contains sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter. The adenovirus 5' sequence containing the 5' ITR and packaging/enhancer region (Ad5 mu 0-1 or bp 1-360) can be employed as the 5' adenovirus sequence in rAd of this invention. The 3' adenovirus sequences including the right terminal (3') ITR sequence of the adenoviral genome spanning about bp 35,353 - end of the adenovirus genome, or map units ~98.4-100 may be desirably employed as the 3' sequence of the rAd. These sequences, which are clearly devoid of the E1 and E4 genes, can flank, or be operatively associated with the minigene in a rAd. Any other necessary Ad gene products will then be supplied by helper viruses and the E1/E4 ORF6 expressing packaging cell of this invention.

Exemplary rAd for use in this invention, for example, may be obtained by homologous recombination of desired fragments from various rAd, a technique which has 35 been commonly employed to generate other rAd for gene

therapy use. In the examples below, a representative rAd, H5.001CBLacZ, is constructed by homologous recombination between the adenovirus dl1004 (also H5dl1004) viral backbone and pAdCBLacZ minigene DNA.

5 H5dl1004 is an Ad5 virus deleted of from about map unit 92.1 through map unit 98, i.e., substantially the entire E4 gene. The dl1004 virus is described in Bridge and Ketner, J. Virol., 632(2):631-638 (Feb. 1989).

The pAdCBLacZ vector is a cDNA plasmid

10 containing Ad m.u. 0-1, an E1 deletion into which is inserted a bacterial  $\beta$ -galactosidase gene under the control of a chicken  $\beta$ -actin promoter, with other regulatory elements as described below, and flanked by Ad m.u. 9-16 and plasmid sequence.

15 The production of the E1/E4 rAd of this invention in the packaging cell line of this invention utilizes conventional techniques. Such techniques include conventional cloning techniques of cDNA such as those described in texts [Sambrook et al, cited above],

20 use of overlapping oligonucleotide sequences of the adenovirus genomes, PCR and any suitable method which provides the desired nucleotide sequence. Standard transfection and co-transfection techniques are employed, e.g., CaPO<sub>4</sub> transfection techniques using the

25 complementation 293 cell line. Other conventional methods employed include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

For example, following the construction and

30 assembly of the desired minigene-containing plasmid vector pAdCBLacZ, the E1/E4 expressing packaging cell line of this invention is infected with the helper virus H5dl1004. The infected cell line is then subsequently transfected with an adenovirus plasmid vector by

35 conventional methods. Homologous recombination occurs

between the E4-deleted H5dl1004 helper and the pAdCBLacZ vector, which permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the rAd. About 30 or more hours 5 post-transfection, the cells are harvested, an extract prepared and the rAd containing the LacZ transgene is purified by buoyant density ultracentrifugation in a CsCl gradient.

### *III. Use of the Recombinant Virus in Gene Therapy*

10 The rAd containing the transgene produced by cooperation of the adenovirus vector and E4 deleted helper virus and packaging cell line, as described above, provides an efficient gene transfer vehicle which can deliver the transgene in a pharmaceutical composition to 15 a patient *in vivo* or *ex vivo* and provide for integration of the gene into a mammalian cell.

20 The rAd are administered to humans in a conventional manner for gene therapy and serve as an alternative or supplemental gene therapy for the disorder to which the transgene is directed. A rAd of this invention may be administered to a patient, preferably suspended in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes 25 sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to those of skill in the art may be employed for this purpose.

30 The rAd are administered in sufficient amounts to transfect the desired target cells, e.g., muscle, liver, epithelial, etc. and provide sufficient levels of transfer and expression of the transgene to provide a therapeutic benefit without undue adverse or with 35 medically acceptable physiological effects which can be determined by those skilled in the medical arts.

Conventional and pharmaceutically acceptable routes of administration include direct delivery to the muscle or other selected cell, intranasal, intravenous, intramuscular, subcutaneous, intradermal, oral and other 5 parental routes of administration. Routes of administration may be combined, if desired.

Dosages of rAd will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. 10 For example, a therapeutically effective human dose of the rAd is generally in the range of from about 20 to about 100 ml of saline solution containing concentrations of from about  $1 \times 10^9$  to  $1 \times 10^{11}$  pfu/ml virus. A preferred human dose is estimated to be about 50 ml saline solution 15 at  $2 \times 10^{10}$  pfu/ml. The dose will be adjusted to balance the therapeutic benefit against any side effects. The levels of expression of the transgene can be monitored to determine the frequency of administration.

An optional method step involves the co- 20 administration to the patient, either concurrently with, or before or after administration of the rAd of a suitable amount of a short acting immune modulator. The selected immune modulator is defined herein as an agent capable of inhibiting the formation of neutralizing 25 antibodies directed against the recombinant vector of this invention or capable of inhibiting or substantially delaying cytolytic T lymphocyte (CTL) elimination of the vector. Among desirable immune modulators are interleukin-12 [European Patent Application No. 441,900]; 30 gamma interferon [S. C. Morris et al, J. Immunol., 152:1047 (1994)]; interleukin-4 [United States Patent No. 5,017,691]; antibody to the CD4 protein, such as anti-OKT 3+ [see, e.g., US Patent No. 4,658,019] or antibody GK1.5 (ATCC Accession No. TIB207); a soluble CD40 molecule or 35 an antibody to CD40 ligand (Bristol-Myers Squibb Co)

[European patent application 555,880, published August 18, 1993]; a soluble form of B7 or an antibody to CD28 or CTLA4 [CTLA4-Ig (Bristol-Myers Squibb Co), European patent application 606,217, published July 20, 1994], or 5 agents such as cyclosporin A or cyclophosphamide. Thus, the pharmaceutical compositions and methods of this invention provide a desirable gene therapy treatment.

#### IV. Recombinant Adeno-Associated Virus

In the following context the term "transgene" means 10 a nucleic acid sequence or reverse transcript thereof, heterologous to the AAV sequence, which encodes a polypeptide or protein of interest. The transgene may be operatively linked to regulatory components in a manner which permits transgene transcription, i.e., the 15 transgene is placed into operative association with a promoter, as well as other regulatory sequences, such as SV40 introns or polyA sequences, useful for its regulation. The composite association of the transgene with its regulatory sequences is referred to herein as a 20 minicassette or minigene.

The composition of the transgene or minicassette sequence will depend upon the use to which the resulting rAAV will be put. For example, one type of transgene sequence includes a reporter sequence, which upon 25 expression produces a detectable signal. Such reporter sequences include without limitation, an *E. coli* B-galactosidase (*LacZ*) cDNA, an alkaline phosphatase gene (ALP) and a green fluorescent protein gene. These sequences, when associated with regulatory elements which 30 drive their expression, provide signals detectable by conventional means, e.g., ultraviolet wavelength absorbance, visible color change, etc.

Another type of transgene sequence includes a therapeutic gene which expresses a desired gene product 35 in a host cell. These therapeutic nucleic acid sequences

typically encode products for administration and expression in a patient *in vivo* or *ex vivo* to replace or correct an inherited or non-inherited genetic defect or treat an epigenetic disorder or disease. Such transgenes 5 may be readily selected by one of skill in this art and the design of the transgene or the minicassette for insertion into the rAAV is not a limitation of this invention.

The term "rAAV" encompasses any recombinant AAV gene 10 therapy vehicle of the prior art, including the AdAAV hybrid virus described in published International Patent Application No. WO96/13598, published May 9, 1996. More specifically, rAAV defines a rAAV comprising: (a) the DNA of at least a portion of the genome of an AAV, which 15 portion is capable of transducing into a target cell at least one selected gene in the absence of cell division; and (b) at least one selected gene (or transgene) operatively linked to regulatory sequences directing its expression, the gene flanked by the DNA of (a) and 20 capable of expression in the target cell *in vivo* or *in vitro*.

Other rAAVs have been described in the art. The method of this invention is not limited by the precise 25 nature of the AAV sequences used in the rAAV, provided that at a minimum both the 5' and 3' AAV inverted terminal repeats are present. Thus, the rAAV may be selected by one of skill in the art, and is not itself a limitation on this invention. The rAAVs specifically disclosed herein are illustrative.

30 By the term "transduction" is meant that the rAAV produced by practice of the invention is capable of infecting a desired target cell and expressing the transgene in the cell by harnessing the cell's machinery. Transduction may include stably integrating the viral DNA 35 into a chromosome of the target cell. "Enhanced

transduction" is defined as the ability of the rAAV in the presence of a conversion agent to transduce the target cell, either *in vitro*, *ex vivo* or *in vivo*, at an efficiency greater than a typical prior art rAAV produced 5 in, and purified from, a culture co-infected with an adenovirus or herpesvirus helper.

This method is based on the observation that the limiting step in rAAV mediated transduction of cells for gene therapy is not the internalization or transfer of 10 the ss viral genome, but rather the subsequent conversion of the single-stranded (ss) viral genome to a transcriptionally active double-stranded (ds) form. Formation of ds DNA intermediates is necessary for recombinant gene expression, which is likely to be 15 modulated by viral and cellular factors through posttranscriptional mechanisms. The inventors have designed a method to overcome this rate-limiting step, thereby enhancing transduction ability of an rAAV and ultimately the use of rAAV in gene therapy protocols.

20 This method of the present invention may employ a conventionally prepared ss rAAV containing a transgene. The prior art produces ss rAAV by co-infection in culture with a helper adenovirus or herpesvirus, followed by purifying the rAAV from the culture contaminants 25 including the helper virus, and infecting the target cell with the rAAV alone. The present invention provides for infecting a target cell with a ss rAAV. However, once the target cell is infected, the infected cell is contacted with an agent which facilitates the conversion 30 of the ss rAAV to the ds form of rAAV. The action of this "facilitating agent" or "conversion agent" causes the ss to ds conversion to occur in the target cell, resulting in enhanced transduction of the recombinant AAV 35 into the target cell. By facilitating the conversion of ss to ds rAAV in the target cell, the method of this

invention may also result in both transduction and stable chromosomal integration of the rAAV into the chromosome of said host cell.

Preferably, for use of this invention the 5 "facilitating or conversion agent" may take several forms.

---

A. The Conversion Agent is a Helper Virus

In one embodiment, the agent is a helper virus and the method includes an additional step of co- 10 infecting the target cell with the helper virus. The helper virus useful in this method contains a selected gene which can facilitate the conversion of ss rAAV to ds rAAV. The selected gene may encode a gene product or 15 polypeptide (or a functional fragment of the polypeptide which shares the biological activity of the full-length polypeptide) which enhances the conversion.

Alternatively, the selected gene may express an antisense or ribozyme which functions in the cell to block or 20 inhibit a cellular gene that normally prevents ss to ds conversion of the rAAV. These genes may also be employed in the second generation rAAV described below.

The helper virus is capable of expressing the selected gene product in the target cell in the absence 25 of cell division. The helper virus may be a wild-type or mutant adenovirus. The helper virus may alternatively be a wild-type or mutant herpesvirus. Preferably, for use as facilitating agents, such viruses are mutants deleted of several normal genes so that the helper viruses and/or 30 their expressed gene products will not cause disease in a patient.

For example, a helper adenovirus useful in this invention may express only a gene product of a single adenoviral early gene. Exposure of the ss rAAV to an Ad 35 early gene product is sufficient to substantially enhance the formation of ds rAAV genome with a coordinate

increase in transduction efficiency. The Ad early genes which are useful in producing this effect are E1, E2a, E4 and functional fragments thereof. However, as demonstrated by the examples below, adenovirus 5 substantially enhances recombinant AAV transduction in vitro in a way that is dependent on expression of the E1 and E4 genes of adenovirus and is directly proportional to the appearance of ds replicative forms of rAAV.

One example of a helper virus is an adenovirus 10 deleted of most of its wild-type early genes and which is capable of expressing only its E4 gene or a functional fragment thereof in the target cell. Among such functional fragments is the ORF 6 of the E4 gene. As described below in the examples, experiments in cell 15 lines indicate that the ORF6 of the adenoviral E4 gene locus is sufficient to significantly enhance rAAV transduction. Selective expression of the E4-ORF6 product of adenovirus accomplishes a increase in transduction efficiency similar to, but somewhat 20 attenuated, compared to that produced by exposure to the E1 and E4 gene products in combination. That is, the ORF6 product of E4 is sufficient to enhance the augmentation of rAAV transduction; but this effect is amplified substantially by E1 gene products.

25 Thus, more preferably, exposure of the rAAV to both the expressed E1 and E4 gene products produces a substantial enhancement of the above-described rate limiting step. Therefore, another exemplary helper virus may also contain more than one gene which, upon 30 expression, facilitates the ss to ds conversion. An example of such a helper virus is an adenovirus which expresses both the E1 and E4 genes, or functional fragments thereof. Still other Ad genes may be expressed by the helper virus, provided that the virus is 35 sufficiently crippled so that it does not cause disease

in the patient contributing the target cells.

Where the agent which facilitates conversion of ss to ds rAAV is a helper virus, the method of the invention comprises co-infecting the target cell with the 5 rAAV and the helper virus. Such co-infection may occur in the context of *ex vivo* therapy, i.e., manipulations performed on cells extracted from the patient, which cells are reinserted into the patient after the method is performed. Alternatively, the patient may be directly 10 co-infected with the two viruses by conventional means. Delivery of the two viruses to the patient may be directed to a specific organ, or to the general circulatory system. Such delivery methods are described in the art for gene therapy of, e.g., cystic fibrosis 15 [see, e.g., US Patent No. 5,240,846].

*B. The Conversion Agent is a Chemical, Drug or Other Entity that can Activate rAAV Transduction*

In another embodiment of the method of this invention, the conversion agent which contacts the cells infected with the rAAV may be selected from the following 20 classes of known compounds or methods: 1) inhibitors of DNA synthesis such as hydroxyurea, hydrogen peroxide, and other direct or indirect inhibitors of DNA polymerase; 2) chemo-therapeutic agents that induce DNA damage, such as 25 cyclophosphamide, alkylating agents, purine analogs, e.g., 6-thioguanine, etc.; 3) drugs that interfere with DNA modifying enzymes, such as inhibitors of topoisomerase, DNA ligase exonucleases and endonucleases; and 4) agents that nonspecifically enhance transcription, 30 such as sodium butyrate, or agents that stabilize cells, such as DMSO. Also, genotoxic agents such as carcinogens may be employed as the conversion agent. Other methods of inducing disruption or damage to DNA may also be 35 useful as agents capable of facilitating ss to ds conversion of rAAV and maybe selected by one of skill in

the art, including physical methods, such as irradiation. These classes of compounds or methods are believed to result in the conversion from ss to ds rAAV.

According to this embodiment of the method of the invention, the rAAV is again produced conventionally, but not co-infected with a helper virus. The ss rAAV is infected into the target cell, and the infected cell is contacted by the agent in an appropriate manner depending on the identity of the agent. These conversion enhancing agents can be employed in *ex vivo* treatment of the target cells infected by the rAAV by application directly to the cells. Such application can occur substantially simultaneously, or consecutively, with application of the rAAV gene therapy vehicle. For example, the infected target cell may be subjected to one of the above-listed compounds or drugs for a desired time period. The parameters for contacting the infected cells with the agent may readily be determined by one of skill in the art. These parameters will depend upon whether the method is performed *ex vivo* or *in vivo*. For example, the number of *ex vivo* infected cells to be treated will be considered for the dosage, and timing of such treatment.

Similarly, the physical status of the patient can determine the parameters of delivery of the agent to the patient *in vivo*. The dosage and amount of the damaging agent may therefore be adjusted by one of skill in the art. Where the agents are typical chemotherapeutic drugs approved for use in humans or animals, such enhanced conversion of rAAV may also occur *in vivo* by the co-administration of the agent, i.e., the chemotherapeutic drug, and the rAAV gene therapy vehicle to the patient. According to this aspect of the invention, the chemotherapeutic drug would be administered only when the rAAV is administered. Appropriate dosages and amounts of chemotherapeutic drugs

and recombinant gene therapy vehicles and means for determining such amounts are within the skill of the art. However, because the effect of the chemotherapeutic drug will enhance the ss to ds conversion of the rAAV and thus 5 enhance its efficiency of transduction into the target cells, it is anticipated that lower dosages than the conventional dosages of either or both the drug and the rAAV could be effectively administered.

10                   C.    Conversion Agent May be Part of the rAAV.  
In still another embodiment of this invention, a novel "second generation" rAAV may be designed to incorporate the conversion agent into the virus, so that both the transgene and the conversion agent are co-expressed in the target cell. Such a novel recombinant 15 adeno-associated virus comprises the following components:

20                   (a) the DNA of at least a portion of the genome of an adeno-associated virus which portion is capable of transducing at least two selected genes or functional fragments thereof into a target cell in the absence of cell division; (b) a first selected gene, i.e., the desired transgene, operatively linked to regulatory sequences directing its expression, and (c) a second selected gene, i.e., the "conversion gene" 25 operatively linked to regulatory sequences capable of directing expression of said second gene. The "conversion gene" upon expression is capable of facilitating the conversion of the ss rAAV to its ds form upon expression. The first and second genes in this rAAV 30 are flanked by the AAV DNA, preferably the 5' and 3' ITRs. An embodiment of such a second generation rAAV is provided schematically in Fig. 11. Its DNA sequence is provided in SEQ ID NO: 5.

35                   Another embodiment of such a novel rAAV may include more than one gene which upon expression has the

ability to facilitate conversion of ss to ds rAAV in the target cell. For example, the novel rAAV described above may also contain an additional selected gene operatively linked to regulatory sequences capable of directing its expression, the additional gene and said second "conversion" gene described above being capable of jointly facilitating the conversion of ss rAAV to its ds form upon expression of both the second and additional genes. In this rAAV, all three genes, i.e., the transgene, the second "conversion" gene and the additional gene are flanked by the AAV DNA.

In one desirable embodiment of a novel rAAVm the AAV ITRs flank a selected transgene, and a conversion gene, which is the adenovirus E4 gene or a functional fragment thereof (e.g., the ORF6 sequence). In another embodiment, the novel recombinant expresses three genes, the transgene, the adenovirus E4 gene or a functional fragment thereof and the adenovirus E1 gene or a functional fragment thereof. The E4 and E1 gene products expressed in the target cell with the transgene, together act to facilitate conversion of the ss to ds form of rAAV.

In still another embodiment of the novel rAAV and its use, the regulatory sequences directing expression of the conversion gene, e.g., whether it be a single second gene or more than a single additional gene, may include an inducible promoter. Thus, expression of the conversion gene occurs only in the presence of an inducing agent. Many inducible promoters and companion inducing agents, e.g., steroids such as glucocorticoids, are known to the art and may be readily selected for incorporation into the rAAV and methods of this invention by one of skill in the art with resort to this description.

35 The method of the invention employing such

"second generation" rAAVs which carry at least one "conversion gene" provides for infecting the target cell with this ss rAAV. Where the promoters directing expression of both the transgene and the conversion gene are constitutive, the infected target cell machinery will direct the expression of the transgene product and conversion gene product. Co-expression in the target cell of the transgene and the "conversion gene" facilitates the conversion of ss rAAV to ds rAAV in the cell, and increases the transduction efficiency, and perhaps stable chromosomal integration, without further method steps.

When the second generation rAAV employed in the method contains the "conversion gene(s)" under the control of inducible promoter(s), the method is slightly altered. Following infection of the target cell by the rAAV, the infected target cell is contacted with a suitable inducing agent, which triggers the inducible promoter to "turn on" production of the conversion gene product. When the inducing agent is removed or stopped, the expression of the conversion gene product is "turned off".

As described above, any prior art rAAV containing a transgene for gene therapy may be used in at least one embodiment of the above methods. The sources, selection and assembly of the various components to generate the rAAV, including the novel rAAV described above, are now conventional and readily accessible to one of skill in this art, given the disclosure contained herein. Such methods employ conventional genetic engineering techniques [See, e.g. Sambrook et al, cited above].

The novel rAAV viruses and the methods of this invention provide efficient gene transfer vehicles for somatic gene therapy and are suitable in pharmaceutical

compositions for *ex vivo* applications and *in vivo* use. When rAAV contain a therapeutic gene, e.g., in place of the LacZ transgene illustrated in the exemplary rAAV, AV.CMVLacZ, by use of the rAAV and the methods described herein, the therapeutic transgene can be delivered to a patient *in vivo* or *ex vivo* to provide for efficient transduction, and possibly stable integration, of the desired gene into the target cell. Thus, these novel rAAV and the methods described herein can be employed to correct genetic deficiencies or defects. The potential of AAV to efficiently integrate its genome into nondividing cells is currently being exploited in the development of gene therapies based on *ex vivo* transduction of hematopoietic stem cells. *In vivo* application of rAAV is primarily being developed for the treatment of CF where purified stocks of virus are instilled into the airway to transduce the terminally differentiated epithelial cells of conducting airway. The methods and compositions described herein can be used with both types of gene therapy. Another condition suitable for such use includes transduction of the low density lipoprotein (LDL) receptor gene into hepatocytes for the treatment of familial hypercholesterolemia. One of skill in the art can generate any number of rAAV which can be used via the above methods for the treatment of these and other disorders.

For *ex vivo* or for *in vivo* therapy, the rAAV may be used to infect the target cells by suspending the virus particles in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to

those of skill in the art may be employed for this purpose.

The rAAV are administered in sufficient amounts to transfect the desired cells and provide sufficient levels of expression of the selected transgene to provide a therapeutic benefit without undue adverse, or with medically acceptable, physiological effects which can be determined by those skilled in the medical arts.

Conventional and pharmaceutically acceptable routes of *in vivo* administration include direct delivery to the target organ, tissue or site, intranasal, intravenous, intramuscular, subcutaneous, intra $\ddot{\text{a}}$ dermal, oral and other parenteral routes of administration. Routes of administration may be combined, if desired.

Dosages of the rAAV for the infecting step of the method will depend primarily on factors such as the therapeutic environment, i.e., *ex vivo* or *in vivo*; the condition being treated, the selected gene, the age, weight and health of the patient, and may thus vary among patients. A therapeutically effective dosage of the rAAV for *ex vivo* treatment will be based upon the multiplicity of infection, which is likely to range from between about 1 to about 10 transducing particles/cell. A therapeutically effective human dosage of the rAAV for *in vivo* infection according to the present invention is believed to be in the range of from about 20 to about 50 ml of saline solution containing concentrations of from about  $1 \times 10^7$  to  $1 \times 10^{10}$  transducing viral particles/ml virus. A preferred human dosage is about 20 ml saline solution at the above concentrations. The dosage will be adjusted to balance the therapeutic benefit against any side effects. The levels of expression of the selected gene can be monitored to determine the selection, adjustment or frequency of dosage administration.

The effective amount of the facilitating agent to be administered is within the skill of the art to determine and will depend upon the identity of the agent. Known dosages of certain of the classes of chemicals and 5 pharmaceuticals described above may be employed in this method to damage the DNA and facilitate ss to ds conversion of the rAAV. Where the agent is a gene expressed by a helper virus, the amounts of infecting virus should be similar to those amounts described above 10 for the rAAV. Of course, where the agent is a gene present in a second generation rAAV, the identical dosages described above for the rAAV will apply.

Several embodiments of the above-described methods of this invention were confirmed in murine models 15 of rAAV mediated gene transfer to both lung and liver. These experiments demonstrated similarly low levels of gene transfer *in vivo* by rAAV, which was increased several orders of magnitude by coinfection with E1 and E4 expressing adenovirus.

20 In summary, experiments were conducted to demonstrate that adenovirus enhances rAAV transduction in cultured cells. During the production and characterization of a lacZ recombinant AAV generated in 293 cells that were coinfecte~~d~~ with an E1 deleted virus, 25 it was observed that purification of rAAV from lysates was associated with substantial loss of lacZ transducing activity when assayed on 293 cells. This drop in rAAV activity was particularly evident in the final step where residual contaminating helper adenovirus was removed by 30 heat inactivation. LacZ transducing activity was recovered by adding adenovirus back to the purified stock of rAAV. These data provided the first indication that adenovirus could substantially enhance the transduction efficiency of rAAV.

As described in Example 10, a series of complementation groups were generated by mixing different adenovirus early gene mutants with purified *LacZ* rAAV, referred to as AV.CMVLacZ (see Example 2). These defined mixtures of viruses were analyzed for *LacZ* transduction on HeLa cells (See Examples 12 and 13). An E1 deletion rAd-H5.CBALP and the E4 deletion mutant dl1004 provided no significant increase in AV.CMVLacZ transduction (Fig. 5A). However, partial activity could be achieved with E1 and E4 mutants that carried less severe deletions. Both dl110 (E1B-55kDa deleted) and dl1010 (ORF6 deleted) enhanced transduction to levels that approached those of Ad5, ts125, and dl802 in terms of the number of positive blue cells, but total  $\beta$ -galactosidase activity was substantially lower (Fig. 5A). These results implicate early regions E1 and E4 in the augmentation of rAAV transduction.

The experiments described below also demonstrate that the novel rAAV which incorporates as its conversion gene, an Ad gene, such as E4, can increase transduction efficiency of the rAAV in the absence of a helper virus. As described in more detail below in Example 15 below, 293 cells were stably transfected with a genomic fragment of Ad5 spanning E4. This E1/E4 expressing cell line and the parent E1 expressing cell line (293) were infected with rAAV and analyzed for transduction. These experiments demonstrated the significance of the combined expression of E1 and E4(ORF6) in the adenovirus mediated augmentation of rAAV transduction.

In the presence of E1 and E4 expression, rAAV transduction was invariably accompanied by the appearance of ds RF monomers and dimers (Example 14). Importantly, the tight correlation between rAAV vector transduction and the accumulation of duplex forms could be achieved in

two different experimental settings; cells infected with E1/E4 expressing adenovirus (Figs. 8A and 8B), or complementing cell lines (Fig. 8C).

The following examples illustrate the 5 construction and testing of the novel packaging cell lines, the E1/E4 deleted rAd of the present invention and the use thereof, improved methods and second generation recombinant AAV production for gene therapy of the present invention. These examples are illustrative only, 10 and do not limit the scope of the present invention.

Example 1 - Novel E1a/E1b and E4 Expressing Packaging Cell Lines

A. Construction of E4 ORF 6 Expressing Plasmids

The entire E4 region from Ad5 or an ORF6 15 minigene were subcloned into a shuttle plasmid that contained a neomycin resistance gene. Two versions of ORF6 minigene were developed that differed in the promoter element. The first used a Zn+2 inducible sheep metallothioneine (MT) promoter to drive ORF 6 expression. 20 The second used a dexamethasone-inducible mouse mammary tumor virus (MMTV) promoter.

An exemplary plasmid useful for the construction of a packaging cell line of this invention is pMMTVE4ORF6. The minigene contained in this plasmid 25 is set out in SEQ ID NO: 1, and contains a mouse mammary tumor virus promoter (MMTV) (nucleotides 1-1506 of SEQ ID NO:1) in transcriptional control of a human E4 ORF 6 gene sequence (nucleotides 1523-2408 of SEQ ID NO: 1), a growth hormone terminator (GH) (nucleotides 2409-3654 of 30 SEQ ID NO: 1), an SV40 origin of replication, plasmid sequences from plasmid pBR322, including a neomycin resistance gene, and an ampicillin resistance gene. The amino acid sequence of ORF 6 is indicated in SEQ ID NO: 2. The various functional fragments of this plasmid may 35 be readily replaced with other conventionally used

sequences and are not critical to the design of the plasmid.

Another plasmid useful for the construction of a packaging cell line of this invention is pMTE4ORF6.

5 The DNA sequence of the minigene contained in this plasmid is similar to that of SEQ ID NO: 1, except that the promoter is a sheep metallothionein promoter (MT promoter) [M. G. Peterson et al, cited above].

A plasmid used as a control for the 10 construction of a packaging cell line of this invention is pLTR.E4(-). This plasmid contains the endogenous constitutive retroviral MLV LTR and most of the Ad E4 gene region except that the endogenous E4 promoter and a portion of E4 ORF1 are missing. The other plasmid 15 sequences remain the same as described above.

Still another plasmid useful for the study of the methods of this invention is pLTR.E4, which contains the constitutive MLV LTR and endogenous E4 promoter and an intact E4 gene. The other plasmid sequences remain 20 the same as described above.

To determine whether ORF6 expression was sufficient to enhance rAAV transduction, the inducible metallothionein (MT)-ORF6 minigene was stably transfected into HeLa cells. This new cell line, HeLa(MT-ORF6) was 25 evaluated for LacZ rAAV transduction in response to ORF6 induction as described below. The cell line 293 (MT-ORF6) expresses ORF-6 of the E4 gene of Ad5 from the metallothionein promoter which is relatively inactive at baseline but can be induced with divalent cations. These 30 293 cells were included to establish the baseline transduction efficiency.

B. *Transfections and Selection of Clones*

Each of the above-described plasmids was transfected by the calcium phosphate precipitation 35 technique into the human embryonic kidney cell line 293

[ATCC CRL1573] which expresses the product of the adenovirus E1 genes, or into HeLa cells, seeded on 100 mm plates (10 µg plasmid/plate). Twenty four hours post-transfection, cells were harvested and seeded at varying dilutions (1:10 - 1:100) in 100 mm plates for about 10 days. Seeding media contain G418 (Geneticin, BRL) at 1 mg/ml. Resistant colonies that developed were selected using the following assays and expanded. Preliminary analysis of clones was based on enhanced transduction efficiency of a recombinant adeno-associated virus, AV.CMVLacZ, and immunofluorescence localization of Ad E4 protein as described in the following examples.

Example 2 - Recombinant AAV and AV.CMVLacZ Transduction Enhancement Assay

15 E1 and E4 Ad gene products are needed for recombinant adeno-associated virus (AAV) function. This primary assay involves seeding the packaging cell lines of Example 1 in 96 well 35 mm culture plates (2x10<sup>6</sup> cells/well) and infecting the cells with purified, heat-treated AV.CMVLacZ at an MOI of 1000 virus particles/cell.

A. Preparation of Recombinant AV.CMVLacZ

20 A recombinant AAV virus was prepared by conventional genetic engineering techniques for the purposes of this experiment. Recombinant AAV was generated by plasmid transfections in the presence of helper adenovirus [Samulski et al, J. Virol., 63:3822-3828 (1989)]. A cis-acting plasmid pAV.CMVLacZ [SEQ ID NO: 4] (see Fig. 10) was derived from psub201 [Samulski et al, J. Virol., 61:3096-3101 (1987)] and contains an *E. coli*  $\beta$ -galactosidase minigene in place of AAV Rep and Cap genes. The 5' to 3' organization of the recombinant AV.CMVLacZ genome (4.9 kb) [SEQ ID NO: 4] includes

25 (a) the 5' AAV ITR (bp 1-173) was obtained by PCR using pAV2 [C. A. Laughlin et al, Gene,

23: 65-73 (1983)] as template [nuc. 53-219];  
(b) a CMV immediate early  
enhancer/promoter [Boshart et al, Cell, 41:521-530  
(1985)] (nuc. 246-839);

5 (c) an SV40 intron (nuc. 856-987);  
(d) *E. coli*  $\beta$ -galactosidase cDNA (nuc.

1039-4512);

10 (e) an SV40 polyadenylation signal (a 237  
Bam HI-BclI restriction fragment containing the  
cleavage/poly-A signals from both the early and late  
transcription units (nuc. 4522-4719) and  
(f) 3'AAV ITR, obtained from pAV2 as a  
SnaBI-BglII fragment (nuc. 4759-4925). All other  
nucleotides are plasmid derived.

15 Rep and Cap genes were provided by a trans-  
acting plasmid pAAV/Ad [Samulski et al, cited above].

Monolayers of 293 cells grown to 90% confluency  
in 150 mm culture dishes ( $5 \times 10^7$  cells/plate) were  
infected with H5.CBALP at an MOI of 10. H5.CBALP (also  
20 called H5.010ALP) is a rAd that contains an alkaline  
phosphatase minigene in place of adenovirus E1a and E1b  
gene sequences (map units 1-9.2 of the Ad5 sequence of  
GenBank [Accession No. M73260]). The alkaline  
phosphatase cDNA is under the transcriptional control of  
25 a CMV-enhanced  $\beta$ -actin promoter in this virus. This  
helper virus is described in Goldman et al, Hum. Gene  
Ther., 6:839-851 (July, 1995); Engelhardt et al, Hum.  
Gene Ther., 5:1217-1229 (October, 1994); and references  
cited therein.

30 Infections were done in Dulbecco's Modified  
Eagles Media (DMEM) supplemented with 2% fetal bovine  
serum (FBS) at 20 ml media/150 mm plate. Two hours post-  
infection, 50  $\mu$ g plasmid DNA (37.5  $\mu$ g trans-acting and  
35 12.5  $\mu$ g cis-acting) in 2.5 ml of transfection cocktail  
was added to each plate and evenly distributed.

Transfections were calcium phosphate based as described [B. Cullen, *Meth. Enzymol.*, 152:684-704 (1987)]. Cells were left in this condition for 10-14 hours after which the infection/transfection media was replaced with 20 ml

5 fresh DMEM/2% FBS. Forty to fifty hours post-transfection, cells were harvested, suspended in 10 mM Tris-Cl (pH 8.0) buffer (0.5 ml/150 mm plate) and a lysate prepared by sonication. The lysate was brought to 10 mM manganese chloride, after which bovine pancreatic

10 DNase I (20,000 units) and RNase (0.2 mg/ml final concentration) were added, and the reaction incubated at 37°C for 30 minutes. Sodium deoxycholate was added to a final concentration of 1% and incubated at 37°C for an additional 10 minutes.

15 The treated lysate was chilled on ice for 10 minutes and solid CsCl added to a final density of 1.3 g/ml. The lysate was brought to a final volume of 60 ml with 1.3 g/ml CsCl solution in 10 mM Tris-Cl (pH 8.0) and divided into three equal aliquots. Each 20 ml sample was

20 layered onto a CsCl step gradient composed of two 9.0 ml tiers with densities 1.45 g/ml and 1.60 g/ml.

Centrifugation was performed at 25,000 rpm in a Beckman SW-28 rotor for 24 hours at 4°C. One ml fractions were collected from the bottom of the tube and

25 analyzed on 293 or 293(E4) cells for *LacZ* transduction. Fractions containing peak titers of functional AV.CMVLacZ virus were combined and subjected to three sequential rounds of equilibrium sedimentation in CsCl. Rotor selection included a Beckman NVT-90 (80,000 rpm for 4

30 hours) and SW-41 (35,000 rpm for 20 hours). At equilibrium, AV.CMVLacZ appeared as an opalescent band at 1.40-1.41 g/ml CsCl. Densities were calculated from refractive index measurements. Purified vector was exchanged to 20 mM HEPES buffer (pH7.8) containing 150 mM

35 NaCl (HBS) by dialysis and stored frozen at -80°C in the

presence of 10% glycerol or as a liquid stock at -20°C in HBS/40% glycerol.

Purified virus was tested for contaminating H5.CBALP helper virus and AV.CMVLacZ titers. Helper virus was monitored by histochemical staining for reporter alkaline phosphatase activity. A sample of purified virus representing 1.0% of the final product was added to a growing monolayer of 293 cells seeded in a 60 mm plate. Forty-eight hours later, cells were fixed in 0.5% glutaraldehyde/phosphate buffered saline (PBS) for 10 minutes at room temperature, washed in PBS (3x10 minutes) and incubated at 65°C for 40 minutes to inactivate endogenous alkaline phosphatase activity. The monolayer was allowed to cool to room temperature, rinsed once briefly in 100 mM Tris-Cl (pH 9.5)/100 mM NaCl/5 mM MgCl, and incubated at 37°C for 30 minutes in the same buffer containing 0.33 mg/ml nitroblue tetrazolium chloride (NBT) and 0.165 mg/ml 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP). Color development was stopped by washing the monolayer in 10 mM Tris-Cl (pH 8.0)/5 mM EDTA. Routinely the purification scheme described above removed all detectable H5.CBALP helper virus by the third round of buoyant density ultracentrifugation.

AV.CMVLacZ titers were measured according to genome copy number (virus particles/ml), absorbance at 260 nm ( $A_{260}$  particles/ml) and LacZ Forming Units (LFU/ml). Virus particle concentrations were based on Southern blotting. Briefly, a sample of purified AV.CMVLacZ was treated with capsid digestion buffer (50 mM Tris-Cl, pH 8.0/1.0 mM EDTA, pH 8.0/0.5% SDS/Proteinase K 1.0 mg/ml) at 50°C for one hour to release virus DNA. The reactions were allowed to cool to room temperature, loading dye was added and electrophoresed through a 1.2% agarose gel. Standard

quantities of ds AV.CMVLacZ genome were also resolved on the gel.

DNA<sub>s</sub> were electroblotted onto a nylon membrane, hybridized with a <sup>32</sup>P random primer labeled restriction fragment, and the resulting blot scanned on a PhosphorImager 445 SI (Molecular Dynamics). A standard curve was generated from the duplex forms and used to extrapolate the number of virus genomes in the sample. LFU titers were generated by infecting indicator cells with limiting dilutions of virus sample. Indicator cells included HeLa and 293 and 293 (E4) lines (described in Example 10 below). Twenty-four hours later, cells were fixed in glutaraldehyde and cells were histochemically stained for *E. coli*  $\beta$ -galactosidase (LacZ) activity as described in J. M. Wilson et al, Proc. Natl. Acad. Sci. USA, 85:3014-3018 (1988). One LFU is described as the quantity of virus that is sufficient to cause visually detectable  $\beta$ -galactosidase expression in one cell 24 hours post-infection.

20        B.    *Induction of ORF6 Expression*

Induction of ORF6 expression with 10  $\mu$ M dexamethasone or 150 $\mu$ M zinc sulfate (for negative control, no inducer used) was initiated 2 hours before the addition of virus and continued throughout the duration of the experiment. Twenty-four hours after the addition of virus, cells were harvested, lysates were generated by sonication and analyzed for the  $\beta$ -galactosidase expression (i.e.,  $\beta$ -galactosidase activity) and virus DNA as described above. Hirt extracts were prepared from low molecular weight DNA from cell extracts. The preparation of the Hirt extracts and subsequent analysis by Southern hybridization were performed by resort to conventional procedures known to one of skill in the art.

35        In the absence of the inducers, the packaging

cell lines generate lower levels of  $\beta$ -galactosidase in rAAV infected cells. Induction of ORF6 expression with the inducer dexamethasone results in a concomitant rise in AV.CMVLacZ cell transduction to a level that was much greater than the parent 293 line. Expression of E1 alone was insufficient to have an effect in the adenovirus mediated augmentation of rAAV transduction.

Results are demonstrated for certain positive clones in the Table I below (see Example 4). However, for 30 cell lines having an MMTV promoter and ORF6 sequence, 4 demonstrated over 90% blue cells illustrative of LacZ production in the presence of dexamethasone, i.e., 293-27-6, 293-27-17, 293-27-18 and 293-27-28.

Example 3 - Immunofluorescence Localization of Ad5 Late Protein.

Positive clones from the assay of Example 2 were infected with the recombinant E4 deleted adenovirus H5dl1004 and screened for E4 complementation using an immunofluorescence assay for late gene expression. The H5dl1004 virus was obtained from Dr. Ketner of Johns Hopkins University and is described in Bridge and Ketner, J. Virol., 632(2):631-638 (Feb. 1989), incorporated by reference herein. Because ORF6 of E4 complements late Ad gene expression, specifically in the formation of the hexon and penton fibers of the adenovirus, cell lines containing ORF6 are able to bind with antibody against these proteins.

Each cell line of Example 1 is infected with E4 deleted virus H5dl1004 virus at an MOI of 0.1. The cells were treated with mouse anti-adenovirus FITC-labeled monoclonal antibody to either the hexon or penton fibers in a 1:10 dilution (Chemicon International Inc., Temecula, CA). Positive clones were identified by reaction with the antibody.

Example 4 - Relative Plaquing Efficiency

The cell lines of Example 1 demonstrating with strong complementation ability in Example 3 were screened for relative plaquing efficiency of H5dl1004 as compared to W162 cells (an E4-complementing Vero cell line which does not express E1) [Weinberg and Ketner, Proc. Natl. Acad. Sci. USA, 80(17):5383-5386 (1983)]. In Table II below, RPE%, i.e., relative plaquing efficiency, represents the titer of H5dl1004 on tested cell lines/titer of H5dl1004 on W162 cells. For example, the RPE of 293 cells is 0.

The positive cell lines selected by all criteria are identified in Table I below, with the results of the assays of Examples 2, 3 and 4.

15

TABLE I

## E1/E4 Double Complementing Cell Lines

	Cell Line	Trans- Gene	Pro- moter	IF/LP	AV. CMV LacZ	RPE%
	293-10-3	ORF6	MT	++++	++++	246
20	293-39-11	ORF6	LTR	++++	+++	52
	293-84-31	E4-	LTR	++++	++++	179
	293-12-31	whole E4	LTR +E4	++++	++++	174
25	293-27-6	ORF6	MMTV		+++++	327
	293-27-17	ORF6	MMTV		++++	313
	293-27-18	ORF6	MMTV		+++++	339
	293-27-28	ORF6	MMTV		++++	261

Example 5 - Construction and Purification of H5.001CBLacZ

30 The plasmid pAd.CBLacZ was constructed as described in detail in K. Kozarsky et al, Som. Cell Mol. Genet., 19(5): 449-458 (1993), incorporated by reference herein. This plasmid contained a minigene comprising a 5'

flanking NheI restriction site, followed by Ad5 sequence m.u. 0-1, followed by an E1 deletion into which is inserted a CMV enhancer/chicken  $\beta$ -actin promoter sequence [T. A. Kost et al, Nucl. Acids Res., 11(23):8287 (1983)], 5 which controls the transcription of the following bacterial  $\beta$ -galactosidase, followed by a poly A sequence and flanked 3' by Ad m.u. 9-16, and another NheI site. In the plasmid, the minigene was flanked on both sides by plasmid sequence containing drug resistance markers.

10 The plasmid pAd.CBLacZ was linearized with NheI and co-transfected by the calcium phosphate co-transfection method into the novel packaging cell line of Example 1 with ClaI digested H5dl1004 (an Ad5 sequence deleted of from about map unit 92.1 through map unit 98, 15 corresponding to substantially the entire E4 gene).

Homologous recombination occurs in the cell line between these two viral constructs between Ad map units 9-16, resulting in rAd, designated H5.001CBLacZ [SEQ ID NO: 3] (Fig. 2). This rAd contains the sequence from 20 pAd.CBLacZ (including Ad map units 0-1 (nuc. 1-330); CMV enhancer/chicken  $\beta$ -actin promoter (CB) (nuucs. 370-928); *E. coli*  $\beta$ -galactosidase (nuucs. 945-4429); the polyA (nuc. 4429-4628); and Ad5 map units 9-92.1 and 97.3 to 100 from H5dl1004 (nuucs. 4671-35408)). This rAd is thereby 25 functionally deleted, and substantially structurally deleted, of the Ad E1 and E4 genes.

30 Viral plaques were selected and screened by the  $\beta$ -galactosidase assay [Wilson (1988), cited above] and H5.001CBLacZ was isolated following three rounds of plaque purification. The purified virus was also subjected to cesium chloride density centrifugation and large scale production.

For the following mouse experiments, virus was used after column purification and glycerol was added to a

final concentration of 10% (v/v). Virus was stored at -70°C until use.

Example 6 - Growth Kinetics of H5.001CBLacZ in Packaging Cell Lines

5 The cell lines identified in Table I were infected with recombinant H5.001CBLacZ at an MOI of .5. The growth kinetics of this virus in the E4 complementing cell lines are shown in Fig. 3. Maximum viral yield is reported as LFU/ml in Table II below.

10

TABLE II

	<u>Cell Line</u>	<u>Maximum Viral Yield</u>
	293-10-3	$2.8 \times 10^{10}$
	293-39-11	$9.5 \times 10^8$
	293-84-31	$1.1 \times 10^9$
15	293-12-31	$4.5 \times 10^8$
	293-27-6	$2.8 \times 10^{10}$
	293-27-17	$2.5 \times 10^{10}$
	293-27-18	$2.9 \times 10^{10}$
	293-27-28	$1.2 \times 10^{10}$

20 When grown in 293-27-18 cells (the E4 ORF6 cell line with MMTV promoter inducible by dexamethasone) the maximum yield of this virus is  $2.9 \times 10^{10}$  LFU/ml. Several of the cell lines were passaged between 5 and 20 times and the viral production of the passages remained stable.

25 However, RPE did fall following repeated passages of cells.

Example 7 - Other Recombinant Adenoviruses

Other related rAds were prepared similarly to H5.001CBLacZ by homologous recombination between 30 pAdCBLacZ and other helper viruses.

As one example, H5.000CBLacZ is a recombinant E1 deleted Ad5 which contains the same minigene as H5.001CBLacZ, but has an intact E4 gene. This rAd was prepared as described by homologous recombination between 35 pAdCBLacZ and a wild-type Ad5.

As another example, H5.010CBLacZ contains the adenovirus map units 0-1, followed by a CMV enhanced, chicken cytoplasmic  $\beta$ -actin promoter, the *E. coli*  $\beta$ -galactosidase gene (lacZ), a polyadenylation signal (pA), and adenovirus type 5 map units 9-100, with a small deletion in the E3 gene (the Ad 5 sub360 backbone). This rAd may be prepared by homologous recombination between the pAdCBLacZ vector and Ad5 virus sub360, which contains a 150 bp deletion within the 14.6 kD protein of the E3 gene. See, e.g., J. F. Engelhardt et al, Proc. Natl. Acad. Sci. USA, 91:6196-6200 (June 1994); and Engelhardt et al, Hum. Gene Ther., 5:1217-1229 (Oct. 1994), both incorporated by reference herein.

These rAds were isolated following transfection [Graham, Virol., 52:456-467 (1974)], and were subjected to two rounds of plaque purification. Lysates were purified by cesium chloride density centrifugation as previously described [Englehardt et al, Proc. Natl. Acad. Sci. USA, 88:11192-11196 (1991)]. Cesium chloride was removed by passing the virus over a BioRad DG10 column using phosphate-buffered saline.

Example 8 - LacZ Gene Transfer into Mouse

A. Transfer into Mouse Muscle

Five to six-week old male C57B/6 mice were 25 anesthetized. Anterior tibialis muscles were exposed and directly injected with either rAd H5.000CBLacZ, H5.010CBLacZ or H5.001CBLacZ as follows: 25  $\mu$ L of purified viral suspension at a stock concentration of 5  $\times$  10<sup>11</sup> virus particles/mL was injected by inserting the tip 30 of the 33 gauge needle of a 100  $\mu$ L Hamilton syringe into the belly of the muscle.

Animals were sacrificed on day 4, 14, 28 and 60 post injection. The muscles were dissected and frozen in liquid nitrogen cooled isopentane. Six  $\mu$ M sections were 35 cut in a cryostat, fixed and stained for  $\beta$ -galactosidase

activity for 6 hours at 37°C.

While the blue stained rAd was found for each virus in the day 4 and day 14 (most abundant) stains, by day 28, the H5.001CBLacZ clearly demonstrated more virus 5 on day 28. By day 60, the only virus which stained positive was the H5.001CBLacZ.

B. Transfer into Mouse Lung and Circulation

RAAd H5.000CBLacZ (control), and H5.001CBLacZ (1x10<sup>11</sup> viral particles) were administered to six week old 10 C57BL/6 female mice by tail vein injection and trachea installation. The animals were sacrificed and their liver and lung tissues were harvested at days 4, 9, 21, 28 and 35 post-administration. The transgene and viral late gene expression were compared.

15 At therapeutic doses of virus, there was diminished expression of late viral proteins at all time points in comparison with transgene.

C. Dose Responses in Liver

Dose responses of E4-deleted and E4 intact rAds 20 in the liver of C57BL/6 mice were studied by tail vein administration of 1.5X10<sup>11</sup>, 5x10<sup>10</sup>, 1.7x10<sup>10</sup>, 5.6x10<sup>9</sup>, and 1.9x10<sup>9</sup> viral particles and comparing the transgene and viral late gene expression at day 4, 21, 28, 35, and 42 post administration.

25 At therapeutic doses of virus, there was diminished expression of late viral proteins at all time points in comparison with transgene.

Example 9 - Other Gene Transfers

A. Human OTC Gene Transfer

30 The human OTC gene [A. L. Horwich et al, Science, 224:1068-174 (1984)] or the human CFTR gene [Riordan et al, Science, 245:1066-1073 (1989)] was used to replace the LacZ as the transgene in the recombinant E1/E4 deleted adenoviruses described above, using the 35 techniques analogous for the construction of the above-

described *LacZ* vectors.

The resulting human OTC-containing rAd were administered at an MOI of 10 to 30 to human hepatocytes. The E1/E4 deleted rAd demonstrated less replication and 5 less late gene expression than when the E1/E4 deleted rAds are administered to muscle, as described in the example above. However, the results of this gene transfer are better than comparable transfers with rAds containing only a deletion in the E1 gene or a deletion 10 in the E1 gene and a point mutation in the E2a gene.

Similar results are demonstrated when the transgene is CFTR and the method of administration is intratracheal into lungs.

Example 10 - Transduction Efficiency of rAAV *LacZ* 15 AV.CMVLacZ) in HeLa Cells Infected with Ad Mutants

A. Viruses

The following viruses were employed in this experiment:

(1) Wild-type Ad 5, propagated in 293 cells; 20 (2) Ad dl110 (an Ad which is deleted of the 55 kb E1B gene) [Babiss et al, *J. Virol.*, **52**(2):389-395 (1984) and Babiss and Ginsberg, *J. Virol.*, **50**(1):202-212 (1984)], propagated in 293 cells,

(3) H5.CBALP (an Ad deleted of its E1A and E1B 25 genes and containing a minigene that expresses alkaline phosphatase from a CMV enhanced  $\beta$ -actin promoter, as described above), propagated in 293 cells,

(4) Ad ts125 (an Ad with a temperature 30 sensitive mutation in the E2A gene which encodes the DNA binding protein) [Ensinger and Ginsberg, *J. Virol.*, **10**(3):328-339 (1972)], propagated in 293 cells,

(5) Ad dl802 (an Ad deleted of its E2a gene), grown in E2A-complementing gmDBP cells as described in Rice and Klessig, *J. Virol.*, **56**(3):767-778 (1985);

(6) Ad dl1004 (an Ad deleted of the E4 gene), grown in E4-complementing Vero W162 cells [Weinberg and Ketner, Proc. Natl. Acad. Sci. USA, 80(17):5383-5386 (1983)] and

5 (7) Ad dl1010 (an Ad deleted of ORF6 of its E4 gene), grown in E4-complementing Vero W162 cells [Weinberg and Ketner, cited above].

All viruses were purified by two sequential rounds of buoyant density ultracentrifugation in CsCl.

10 B. Experimental Procedures

HeLa cells seeded in 6 well, 36mm culture plates ( $2 \times 10^6$  cells/well) were infected with wild-type Ad5 or an adenovirus early gene mutant as described in Part A at an MOI of 10pfu/well. Infections were done in 15 1.0 ml DMEM/2%FBS. Six hours post-infection, monolayers were washed and 1.0 ml fresh DMEM/2% FBS media containing AV.CMVLacZ at  $4 \times 10^9$  virus particles/ml were added. Although the AV.CMVLacZ virus lot used in these 20 experiments was shown to be free of H5.CLALP helper virus by histochemical staining, the virus sample was subjected to heat treatment (60°C for 20 minutes) prior to use to ensure the absence of contaminating adenovirus. Two hours later, 1.0 ml of DMEM/115% FBS was added to each well.

25 Twenty-four hours after the addition of AV.CMVLacZ, cells were harvested. Each test condition was done in triplicate to enable virus transduction to be evaluated in terms of three outputs: histochemical staining for  $\beta$ -galactosidase activity (below), 30 intracellular  $\beta$ -galactosidase specific activity (Example 11), and the molecular form of the virus DNA (Example 12).

HeLa cells were histochemically stained for *E. coli*  $\beta$ -galactosidase (LacZ) activity as described in J. 35 M. Wilson et al, Proc. Natl. Acad. Sci. USA, 85:3014-3018

(1988). The different combinations that were tested included cells transfected with AAV vector alone (AV.CMVLacZ), vector plus wild-type Ad5 (+Ad5), vector plus dl110 (+dl110), vector plus Ad mutant H5.CBALP (+H5.CBALP), vector plus Ad mutant ts125 (+ts125), vector plus Ad mutant dl802 (+dl802), vector plus Ad mutant dl1004 (+dl1004), and vector plus Ad mutant dl1010 (+dl1010).

The results were observed in photomicrographs at magnification 10X (not pictured) of histochemical stains for recombinant  $\beta$ -galactosidase activity. The results indicated that wild-type Ad5 and the E2a mutants ts125 and dl802 caused a significant increase in LacZ rAAV transduction as measured by the number of positive blue cells and the degree of stain intensity. Both dl110 (E1B-55kDa) and dl1010 (ORF6) enhanced transduction to levels that approached those of Ad5, ts125, and dl802 in terms of the number of positive blue cells.

The E1 deletion recombinant H5.CBALP provided no significant increase in AV.CMVLacZ transduction. Expression of E1 alone was insufficient to have an effect in the adenovirus mediated augmentation of rAAV transduction as evidenced by lack of significant increase in transduction obtained with HeLa cells infected with the E4 deletion mutant dl1004. A significant drop in transduction occurred following removal of ORF6 from the E4 region from the coinfecting adenovirus (Fig. 5A).

It is believed that these results demonstrate that the adenoviral gene products, E4 and E1 indirectly promote the formation of ds DNA intermediates that are transcriptionally active.

Example 11 - Quantitation of Enhanced Vector Transduction

(A) A duplicate set of HeLa cells as described in Example 10B were used in this experiment. Twenty-four hours after the addition of AV.CMVLacZ recombinant, for

intracellular  $\beta$ -galactosidase assays, cell pellets were suspended in 0.5 ml PBS and sonicated. Cell debris was removed by centrifugation (15,000Xg for 10 minutes) and the clarified extract assayed for total protein [M. Bradford, Anal. Biochem., 72(1-2):248-254 (1976) and M. Bradford et al, Fed. Proc., 35(3):274 (1976)] and  $\beta$ -galactosidase activity [Sambrook et al., cited above] using *o*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) as substrate.

10 Fig. 5A demonstrates the transduction efficiency quantitated by measuring  $\beta$ -galactosidase enzyme activity in the lysates from infected HeLa cells and also assayed for total protein. In Fig. 5A, the test condition is shown along the horizontal axis, and

15 intracellular  $\beta$ -galactosidase specific activity (milliunits/mg protein) using ONPG as substrate is plotted on the vertical axis. Below each bar, the fold-induction in specific activity relative to cells that received the AV.CMVLacZ vector alone is given.

20 The results of Fig. 5A demonstrate that the E2a mutants ts125 and d1802 produced 134-fold and 225-fold increases in  $\beta$ -galactosidase activity, respectively, as compared to that achieved with purified rAAV alone. In comparison, cells infected with wt Ad5 generated 107-fold increase in  $\beta$ -galactosidase activity.

25 (B) In another experiment, HeLa cells ( $2 \times 10^6$ ) were infected with increasing multiplicities of wild-type Ad5 or the E2 mutant d1802. Six hours post-infection, monolayers were washed and infected with AV.CMVLacZ at 30 1000 virus particles/cell. Twenty-four hours after the addition of AV.CMVLacZ, cells were harvested and assayed for total protein and  $\beta$ -galactosidase activity.

35 The results are illustrated in the bar graph of Fig. 5B, in which adenovirus MOI's are given along the horizontal axis, and intracellular  $\beta$ -galactosidase

specific activity along the vertical axis. Enhancement of rAAV transduction was proportional to input helper adenovirus from MOIs of 1 to 50 for both wild type Ad5 and dl802. Higher doses of virus were cytopathic,  
5 leading to a fall in  $\beta$ -galactosidase expression. Enhanced transduction was achieved when the cells were infected prior to, or at the time of, rAAV infection. The E1 deletion recombinant H5.CBALP and the E4 deletion mutant dl1004 provided no significant increase in  
10 AV.CMVLacZ transduction. Both cells infected with dl110 (E1B-55kDa) and with dl1010 (ORF6) demonstrated substantially lower total  $\beta$ -galactosidase activity than those infected with Ad5, ts125, or dl802.

Example 12 - Analysis of Low Molecular Weight DNAs in  
15 AV.CMVLacZ Transduced Cells

Studies with these early gene mutants of adenovirus suggested that expression of adenoviral genes rather than the virion itself was responsible for enhancement of rAAV transduction. To further investigate these mechanisms  
20 and to determine if conversion of ss to ds genome limits the transduction efficiency of rAAV, the molecular state of the rAAV genome was characterized in the infected cells. The relationship between RFm formation and lacZ rAAV transduction was explored in experiments where the  
25 dose of coinfecting virus was varied (MOI=1, 5, or 10).

(A) A duplicate set of HeLa monolayers as described in Example 10 were harvested 24 hours after they were transduced with the recombinant AV.CMVLacZ and cultured with or without helper adenovirus.

30 Episomal DNA was extracted from cell pellets using a modification of the procedure originally described by B. Hirt, J. Mol. Biol., 26:365-369 (1967). Briefly, cells were suspended in 320 ml Tris-Cl (pH8.0)/10 mM EDTA and SDS added to a final concentration  
35 of 1%. The mixture was incubated at 37°C for 30 minutes.

Pronase and proteinase K were added to final concentrations of 500  $\mu$ g/ml and 20  $\mu$ g/ml, respectively, and the reaction incubated at 37°C for 2 hours. Sodium chloride was added to a final concentration of 1.1 M and 5 incubated at 4°C overnight. The precipitate that developed during the 4°C incubation was pelleted at 20,000  $\times g$  for 30 minutes and the clear supernatant carefully removed. The supernatant was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) followed 10 by chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with ethanol. The final pellet was suspended in 50  $\mu$ l Tris-Cl (pH 8.0)/1.0 mM EDTA.

These Hirt extracts were analyzed by Southern blot hybridization. Samples (5  $\mu$ l) of each Hirt extract 15 were resolved through a 1.2% agarose gel, electroblotted onto a nylon membrane and hybridized with a  $^{32}P$  random primer labeled cDNA of the SV40 polyA signal used in AV.CMVLacZ.

An autoradiogram of the experiment of Example 20 12 (not pictured), identifies and labels bands corresponding to the ss AV.CMVLacZ genome (SS), a monomer replicative form (RFm), and concatamer replicative forms (RFd). Bands corresponding to the ss AV.CMVLacZ genome (SS), a monomer replicative form (RFm), and concatamer 25 replicative forms (RFd) were identified and labeled. To reference the RFm band, a plasmid carrying AV.CMVLacZ was digested to release the entire genome. Autoradiogram exposure times were 14 hours and 69 hours.

In this autoradiogram, the full spectrum of 30 molecular species present during a lytic infection was demonstrated in cells infected with both LacZ rAAV and wild type adenovirus. Both the input ss genome (SS) and monomeric and dimeric forms of ds replicative intermediates (RFm and RFd) are present. This contrasts 35 with cells infected with purified rAAV alone, where ss

5 genome is the sole molecular form detected. Analysis of cells coinfecte~~d~~ with the adenovirus early gene mutants revealed a direct correlation between formation of ds forms of the rAAV genome and the enhancement of *LacZ* transduction. Mutant adenoviruses that were ineffective in enhancing rAAV transduction (i.e., the E1 deleted mutant H5.CBALP and the E4 deleted mutant dl1004) failed to promote the formation of ds forms of AAV.

10 Cells infected with adenovirus deleted of E2a (dl802) or partially deleted of E1 (dl110) or E4 (dl1010) additionally demonstrated a band whose size was identical to the ds replicative monomer (RFm) of the lacZ rAAV genome and whose abundance correlated directly with the expression of  $\beta$ -galactosidase activity (compare results 15 of Example 14 to these described results). Slower migrating concatomers, likely dimers, of duplex rAAV were also detected in the autoradiogram described above.

20 In the presence of E1 and E4 expression, rAd transduction was invariably accompanied by the appearance of ds RF monomers and dimers.

25 The high molecular weight band in sample lane +H5.CBALP is helper virus DNA. Helper virus DNA is recognized by the SV40 probe because the CBALP minigene also utilizes the SV40 polyA signal.

(B) In another experiment, HeLa cells were infected with wt Ad5 or the E2 deleted mutant dl802 as described in Example 10B. Monolayers were harvested 24 hours later and analyzed for  $\beta$ -galactosidase activity and RFm synthesis. Monomer bands similar to those shown in the 30 autoradiogram described above were quantitated on a PhosphorImager 445 SI and assigned values (CPM).

35 The results are illustrated in the graphs of Figs. 8A and 8B, in which  $\beta$ -galactosidase specific activity and CPM are plotted along the vertical axis of each figure. Adenovirus MOI's are given on the horizontal

axis of each figure. Data obtained from low MOI infections (1, 5, and 10) are shown. Importantly, the tight correlation between rAAV vector transduction and the accumulation of duplex forms could be achieved in 5 cells infected with E1/E4 expressing adenovirus. The level of  $\beta$ -galactosidase and abundance of RFm increased in proportion to the amount of infecting wild-type Ad (Fig. 6A) and dl802 (Fig 6B). These data suggest that synthesis of an episomal duplex intermediate is an 10 obligatory event in transduction.

Example 13 - Duplex End-Analysis

The following is a description of a model for leading strand synthesis of a complementary AAV strand in the presence of Rep (+Rep) or absence of Rep (-Rep). 15 Refer to Figs. 7A-7F. Rep expresses a terminal resolution activity that can convert a duplex structure with closed-ends to an open-ended duplex. In the absence of Rep, terminal resolution is impaired leaving the covalently closed, hairpin structures intact. Under 20 these conditions, hairpins are expected to be found leftward and rightward, since both strands of a rescued ds AAV genome are packaged into virions. Figs. 7B-7F are a flow chart demonstrating the strategy for identifying the terminal structure of duplex RFm that is synthesized 25 from ss AV.CMVLacZ in response to adenoviral gene expression.

Fig. 7C illustrates a closed end and an open end fragment of rAV.CMVLacZ. Figs. 7D, 7E and 7F indicate the mixture of open-ended and covalently closed duplex 30 fragments generated by NotI digestion at position 4509 in the absence of terminal resolution. The NotI 4509 digestion provides a convenient means of releasing a 361 bp fragment that contains the right ITR in the context of a hybridization target (i.e. SV40 pA). In the presence 35 of terminal resolution, only the open-ended 361 bp

fragment would be expected to be generated (Fig. 7D) by such digestion.

The resulting electrophoretic gel (not pictured), revealed in lane (1) the results of digestion of a 5 plasmid carrying an AV.CMVLacZ cDNA to release the rAAV vector, and subsequent digestion with NotI to release the right terminal 361 bp fragment. In lane (2) a sample of NotI digested Hirt DNA extracted from HeLa cells infected with wild-type Ad5 and transduced with AV.CMVLacZ 10 resulted in the release of two fragments, labeled FormI and FormII. (See, also, Figs. 8A and 8B). The migration of ss AV.CMVLacZ (SS) and RFm were also seen.

The ds AV.CMVLacZ intermediates that accumulated in cells infected with adenovirus were likely the result of 15 leading strand DNA synthesis, initiating from the duplex region of the vector ITR. In the absence of Rep, this conversion event was anticipated to generate molecules in which one end is open and the other is covalently closed (Fig. 7A). To further characterize the structure of this 20 ds intermediate Hirt extracts from cells coinfecte with rAV.CMVLacZ and Ad5 were digested with NotI to release the termini of the ds intermediate which, if left open, would be approximately 361 bp in length. The resulting filters were hybridized with a probe specific for the 25 SV40 polyadenylation signal positioned immediately upstream of the rightward ITR. At least two forms were released from the right end of duplex genomes, one that migrated to a position in the gel that predicted an open-ended conformation (Form II), and a second slower 30 migrating species (Form I). Although this result was consistent with the model (Figs. 7A-7F), it was difficult to predict with certainty the structure of Form I. Its retarded mobility did, however, suggest a conformation that differed from the open-ended Form II.

Example 14 - Analysis of AV.CMVLacZ Transduction Efficiency in 293 Cells Stably Transfected with an Inducible E4 ORF6 cDNA

Cell lines used in this assay were prepared as 5 described in Example 1. 293(MT-ORF6) cells and HeLa(MT-ORF6) cells were seeded in 6 well 35 mm culture plates (2x10<sup>6</sup> cells/well) and infected with purified, heat-treated AV.CMVLacZ at an MOI of 1000 virus particles/cell. Induction of ORF6 expression with from 10 none to increasing concentrations of zinc sulfate was initiated 2 hours before the addition of virus and continued throughout the duration of the experiment.

Twenty-four hours after the addition of virus, cells were harvested, lysates were generated by sonication and 15 analyzed for the  $\beta$ -galactosidase expression (i.e.,  $\beta$ -galactosidase activity) and virus DNA as described in the preceding examples. Hirt extracts were prepared from low molecular weight DNA from cell extracts. The preparation of the Hirt extracts and subsequent analysis 20 by Southern hybridization were performed similarly to those described in the examples above.

The results of this experiment were as follows:

(1) Specific Activity

The results are illustrated in the bar 25 graph of Fig. 8A. Specific activity (milliunits  $\beta$ -galactosidase/mg protein) is plotted along the vertical axis. Below each bar is given the concentration of zinc used for induction, the fold-induction relative to 293 cells, and the fold-induction relative to 293(ORF6) cells 30 maintained in the absence of zinc. As shown in Fig. 8A, in the absence of Zn<sup>+2</sup>, the 293(MT-ORF6) cell line generated 39-fold higher levels of  $\beta$ -galactosidase in rAAV infected 293 cells. Induction of ORF6 expression 35 with increasing amounts of Zn<sup>+2</sup> resulted in a concomitant rise in AV.CMVLacZ cell transduction to a level that was

445-fold greater than the parent 293 line. Expression of E1 alone was insufficient to have an effect in the adenovirus mediated augmentation of rAAV transduction.

The specific activity of  $\beta$ -galactosidase 5 was 196.2 mUnits/mg in E1/E4 expressing 293 cells, compared to 1.0 mUnit/mg in 293 cells that only expressed E1 genes. These experiments support a mechanism for enhancing rAAV transduction that is dependent on the combined expression of both E1 and E4 adenoviral genes.

10 (2) Molecular Analysis of the AV.CMVLacZ  
Genome

The duplex monomer replicative form (RFm) was quantitated and the values (CPM) plotted along the vertical axis in the bar graph of Fig. 8B. The 15 concentration of zinc used for induction and the fold-induction relative to 293(ORF6) cells maintained in 0 mM zinc is given below each bar.

An autoradiogram (not pictured) shows the agarose gel resolved Hirt extracts from the AV.CMVLacZ 20 transduced cells described above. A plasmid carrying the AV.CMVLacZ cDNA was digested to release the entire sequence and loaded in a lane of the autoradiogram. The band that appeared in this lane therefore reflected the migration of a monomer duplex replicative form (RFm). 25 The migration of the ss AV.CMVLacZ genome (SS), RFm, and dimers of the duplex replicative form (RFd) were also shown. Lanes of the autoradiogram labeled (0), (50), (100), (150), (200), and (250) contained samples from 293(MT-ORF6) cells that were induced with the indicated 30 concentration of zinc. A Hirt extract from 293 cells (lane labeled 293) transduced with AV.CMVLacZ was also shown.

35 Analysis of Hirt extracts revealed the presence of the RFm in the rAAV infected 293(MT-ORF6) cells that was not present in similarly infected 293

cells. When the induction profiles (Figs. 8A and 8B) that describe AV.CMVLacZ transduction efficiency were compared, the results were plotted in Fig. 8C. Specific activity (milliunits  $\beta$ -galactosidase/mg protein) data from Fig. 8A and counts-per-minute data (CPM) of AV.CMVLacZ RFM from Fig. 8B are plotted along the vertical axis, and concentration of zinc sulfate used during the experiment is shown along the horizontal axis.

The two profiles are near mirror images.

Importantly, the RFM increased in proportion to the increment in lacZ transducing activity that occurred as ORF-6 expression was induced with  $Zn^{+2}$  (Fig. 8C). Similar results were obtained with a 293 derived cell line that expresses ORF6 from the glucocorticoid responsive MMTV promoter.

Example 15 - Enhanced AV.CMVLacZ Transduction in HeLa Cells Carrying an Inducible ORF6 Minigene

HeLa(MT-ORF6) cells ( $2 \times 10^6$ ) were transduced at an MOI of 1,000 AV.CMVLacZ recombinant particles/cell in absence of zinc sulfate inducer or in the presence of 50, 100, 150, 200, or 250  $\mu M$  zinc sulfate inducer in the media during transduction. Twenty-four hours later, cells were harvested, cell extracts were prepared by sonication, and analyzed for transgene expression (i.e.,  $\beta$ -galactosidase activity). Cell monolayers were histochemically stained for  $\beta$ -galactosidase activity.

The resulting photomicrographs (not pictured) illustrated that histochemical staining revealed an increase in the number of cells scored lacZ positive as the concentration of  $Zn^{+2}$  in the medium was raised from 0 to 200 mM. Concentrations of 250 mM zinc were found to be toxic to the cells.

Specific activity (milliunits  $\beta$ -galactosidase/mg protein) is plotted in Fig. 9 along the vertical axis. Below each bar is given the concentration of zinc used

for induction, the fold-induction relative to HeLa cells, and the fold-induction relative to HeLa(Mt-ORF6) cells maintained in the absence of zinc. Histochemical staining revealed an increase in the amount of 5  $\beta$ -galactosidase in lysates as the concentration of  $Zn^{+2}$  in the medium was raised from 0 to 200 mM.

Example 16 - Southern Blot Analysis of Low Molecular Weight DNAs from AV.CMVLacZ Transduced HeLa(MT-ORF6) Cells

10 Following Induction of E4ORF6

Hirt extracts were prepared from HeLa(MT-ORF6) cells transduced with AV.CMVLacZ as described in Example 15 in the presence of increasing concentrations of  $Zn^{+2}$  to determine whether synthesis of duplex intermediates 15 contributed to the augmentation in AV.CMVLacZ transduction.

20 Samples of HeLa(MT-ORF6) cells that were induced with a concentration of zinc sulfate (0, 50, 100, 150, 200, and 250) were resolved on a 1.2% agarose Southern gel (not pictured), transferred to a nylon membrane, and hybridized with a LacZ-specific probe. One lane contained a plasmid encoding AV.CMVLacZ that was digested to release the entire genome. Bands corresponding to the ss AV.CMVLacZ genome (SS), duplex monomers (RFm), and 25 duplex dimers (RFd) were indicated on the gel.

20 Southern analysis indicated that Hela and uninduced Hela(MT-ORF6) cells demonstrated a single band on Southern blots which comigrated with the ss genome. Induction of ORF-6 resulted in the appearance of 30 detectable levels of ds monomer but only at higher concentrations of  $Zn^{+2}$ . A band comigrating with the RFd was present in all cell preparations, the relevance of which is unclear since the monomer is a likely precursor to the dimer.

Example 17 - Effect of Adenovirus Infection on In Vivo AV.CMVLacZ Targeting Efficiency To Murine Liver

5 The impact of adenoviral gene expression on rAAV transduction in murine liver was studied by sequentially infusing into the portal vein early gene mutants of adenovirus followed by rAAV.

Balb/c mice, 4- to 6-weeks old [Jackson Laboratories, Bar Harbor, Maine] were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg) and 10 xylazine (10 mg/kg). For liver studies, a 1 cm left flank incision was made and the spleen exposed.

15 Samples of purified, heat-treated AV.CMVLacZ in 50  $\mu$ l HBS ( $1 \times 10^{11}$  virus particles) were used alone or spiked with helper adenovirus containing  $2 \times 10^{10}$  A<sub>260</sub> particles of purified dl1004, H5.CBALP, or ts125 in a final volume of 50  $\mu$ l. The dose of adenovirus was sufficient to transduce >25% of hepatocytes. The virus mixture was injected just beneath the splenic capsule and the abdomen was closed with 3-0 vicryl.

20 Necropsies were performed 3 days post-infusion and tissue frozen in O.C.T. embedding compound. Frozen sections (6 $\mu$ m) (LacZ+ALP) were prepared and histochemically stained for  $\beta$ -galactosidase enzyme and alkaline phosphatase activity. Sections were 25 counterstained with neutral red and mounted.

A  $\beta$ -galactosidase positive hepatocyte targeted with AV.CMVLacZ at magnification 20X was obtained. Histochemical analyses of liver tissue harvested 3 days after gene transfer demonstrated that administration of 30  $10^{11}$  particles of purified rAV.CMVLacZ alone into the portal vein was not associated with appreciable gene transfer (<0.01% of cells), confirming the inherent inefficiency of the rAAV system.

35 Preinfusion with E4 deleted virus had no impact on rAAV transduction in mouse liver, whereas E1 deleted

virus demonstrated a modest increment in lacZ positive hepatocytes to about 0.1%. The most significant increase in rAAV transduction occurred following infusion of the E2a adenovirus mutant ts125 with lacZ expression detected 5 in 10-25% of hepatocytes. A direct relationship between adenovirus gene expression and rAAV transduction was demonstrated in animals infused with both lacZ-rAAV and the ALP expressing E1 deleted virus. The dose of adenovirus was reduced 10-fold to minimize the 10 coincidental occurrence of coinfection. Histochemical studies demonstrated co-localization of ALP and  $\beta$ -galactosidase in the majority of  $\beta$ -galactosidase expressing hepatocytes.

Example 18 - Effect of Adenovirus Infection on In Vivo AV.CMVLacZ Targeting Efficiency To Murine Lung

Experiments described in Example 17 for mouse liver were adapted for the study of rAAV mediated gene transfer to mouse lung. For lung experiments, anesthetized Balb/C animals were intubated as described in DeMatteo et al, 20 Transplantation (Baltimore), 59(5):787-789 (1995). Briefly, a midline 2 cm skin incision was made in the neck to expose the trachea. A 2 inch 18 gauge angiocatheter was passed through the mouth, positioned in the midportion of the trachea, and connected to a rodent 25 ventilator (#55-3438 Harvard). Polyethylene (PE#10, Intramedic) was fed through the catheter via a side port and advanced beyond the tracheal bifurcation. Using a Hamilton syringe, virus samples (30 $\mu$ l) were slowly infused into the lung through the polyethylene tubing. 30 Samples contained the same formulation of purified, heat-treated AV.CMVLacZ with or without helper adenovirus, as described for liver injections.

Tissue was harvested 72 hours post-infusion. Frozen sections were histochemically stained for  $\beta$ -galactosidase 35 activity and counterstained with neutral red.

Frozen sections from lung (AV.CMVLacZ) showed a  $\beta$ -galactosidase positive airway epithelial cell targeted with AV.CMVLacZ. Similar studies were performed in the murine model of lung-directed gene transfer.

5 Adenoviruses were instilled into the trachea prior to the instillation of rAAV. Analysis of lung tissue 3 days later revealed only a rare  $\beta$ -galactosidase positive cell in animals instilled with rAAV alone. No detectable enhancement of rAAV transduction was noted in animals  
10 preinstilled with adenovirus deleted of either E1 or E4. Substantial enhancement of transduction was achieved in conducting airway and alveolar cells of animals administered the E2a mutant adenovirus.

These experiments in murine models of gene therapy  
15 directed to liver and lung verified that the efficiency of rAAV transduction is low due limited conversion of the input ss genome to a transcriptionally active ds intermediate, and that this conversion is facilitated by expression of adenovirus E1 and E4 gene products.

20 Example 19 - Second Generation rAAV with Regulated Minigene Capable of Enhancing Transduction

The experiments described in previous examples illustrated the following principles: 1) purified rAAV is a relatively inefficient gene transfer vehicle *in vitro* and *in vivo* and 2) the rate limiting step in transduction is not viral entry but rather conversion of the virion's ss DNA genome to a transcriptionally active ds DNA genome. Adenovirus can substantially enhance transduction through expression of a subset of its genes.  
25 It does this by promoting conversion of the virion's genome to its ds form. One approach to accomplish this is to incorporate into the recombinant AAV genome a minigene that expresses the minimal adenoviral genes necessary to enhance transduction, i.e., the ORF6 region  
30 of E4.  
35

Two approaches have been considered in designing this modified rAAV. The first strategy is based on a rAAV genome that has two transcriptional units in series, one expressing the therapeutic gene and the other 5 expressing its E4 ORF6 from a constitutive promoter. While this may, in fact, be useful in many situations, ~~constitutive expression of ORF6 may be detrimental to the cell and potentially could elicit a destructive immune response.~~

10 The second version of this rAAV includes the therapeutic minigene in addition to the ORF6 transcriptional unit which, in this case, is expressed from an inducible promoter. When this second gene rAAV is administered to the cells (ex vivo strategies) or to 15 the patient (in vivo strategies), the inducing agents are administered at the time of gene transfer or soon thereafter. If the ds genomic form or its integrated derivative is stable, the induction of ORF6 will only be necessary at the time of gene transfer into the recipient 20 cell. Following this, its inducing agent will be withdrawn and the ORF6 gene will be turned off.

An rAAV that illustrates this concept of inducible ORF6 has been constructed and tested *in vitro*. A schematic of the vector pAV.CMVALP.GRE-ORF6, is shown in 25 Fig. 11 and its sequence is illustrated in SEQ ID NO: 5. This second generation construct contains flanking 5' and 3' AAV ITR sequences. The human placental alkaline phosphatase cDNA (ALP) is included in a minigene in which the promoter from the immediate early gene of 30 cytomegalovirus drives the transcription. A second transcriptional unit is cloned between the ITRs in series and in direct orientation with the alkaline phosphatase minigene. The second transcriptional unit expresses the 35 Ad5-E4-ORF6 from a glucocorticoid dependent promoter (GRE) with an SV40 polyadenylation signal. This is

called a second generation rAAV construct.

Specifically, pAV.CMVALP.GRE-ORF6 [SEQ ID NO: 5] generates a novel rAAV containing the *LacZ* transgene and the Ad E4 ORF 6 which facilitates ss to ds conversion of rAAV. The plasmid includes a flanking AAV 5' ITR sequence (nucs. 53-219); CMV enhancer/promoter (nucs. 255-848); human placenta alkaline phosphatase cDNA (ALP) (nucs. 914-2892); SV40 polyA (nucs. 2893-3090); GRE promoter (nucs. 3114-3393); Ad5 E4-ORF6 cDNA (nucs. 3402-4286); SV40 polyA (nucs. 4315-4512); and 3' AAV ITR (nucs. 4547-4713). All other nucleotides are plasmid derived.

The second generation rAAV construct was used to produce and purify rAAV virions which were exposed to HeLa cells that were left untreated or incubated with dexamethasone. In the absence of dexamethasone, (a condition under which little ORF6 should be expressed), little transduction was observed as measured by expression of the alkaline phosphatase gene. Cells incubated in dexamethasone expressed in ORF6 gene and the transduction efficacy was enhanced at least 5-fold. This provides evidence to support that a gene product expressed from the rAAV can function in cis to enhance expression of the transgene.

**25 Example 20 - Application to Bone Marrow Directed Gene Therapy**

Bone marrow directed gene therapy represents the paradigm of *ex vivo* gene therapy where the target cell is the hematopoietic stem cell. The basic strategy is to incorporate (i.e., integrate) a therapeutic minigene into the chromosomal DNA of hematopoietic stem cells which are transplanted into a recipient patient whose own bone marrow has been ablated allowing repopulation of its lymphohematopoietic system with progeny of the genetically corrected stem cell.

The problem with this approach has been efficiently transfecting genes into stem cells. Most studies of bone marrow directed gene therapy have utilized recombinant retroviruses which have not been very efficient. One 5 problem is that retroviruses integrate their provirus only when the target cell is dividing. Unfortunately, most stem cells ~~in-vitro~~ are quiescent and not dividing. rAAV holds the promise of integrating the provirus more 10 efficiently into non-dividing stem cells. However, purified rAAV is not very efficient with respect to integration when used alone. In cultured cells, integration is observed in less than 1% of the cells. The same conditions that activate the conversion of ss to 15 ds genome also enhance the integration of the ds intermediate into the chromosomal DNA.

Therefore, a desirable application of the methods and compositions of this invention is in bone marrow directed gene therapy. According to this method, stem 20 cells are genetically modified with rAAV and an inducing agent *ex vivo* using the constructs and methods described above (see e.g., Example 19). Genetically modified stem cells are subsequently transplanted by conventional techniques.

Numerous modifications and variations of the present 25 invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention, such as selections of different transgenes and plasmids 30 for the construction of the packaging cell lines and rAds, or selection or dosage of the viruses or immune modulators, are believed to be encompassed in the scope of the claims appended hereto.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Trustees of the University of Pennsylvania  
Wilson, James M.  
Fisher, Krishna J.  
Gao, Guang-Ping

(ii) TITLE OF INVENTION: Recombinant Adenovirus and Adeno-  
Associated Virus, Cell Lines,  
and Methods of Production  
and Use Thereof

(iii) NUMBER OF SEQUENCES: 5

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Howson and Howson  
(B) STREET: Box 457, 321 Norristown Road  
(C) CITY: Spring House  
(D) STATE: PA  
(E) COUNTRY: USA  
(F) ZIP: 19477

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release 1.0 Version 1.30

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: WO  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/462,014  
(B) FILING DATE: 05-JUN-1995

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/549,489  
(B) FILING DATE: 27-OCT-1995

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Bak, Mary E.  
(B) REGISTRATION NUMBER: 31,215  
(C) REFERENCE/DOCKET NUMBER: GNVNP012CIPPCT

## (ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (215) 540-9206  
(B) TELEFAX: (215) 540-5818

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3653 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1521..2405

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCATGTGT CAGAGGTTT CACCGTCATC ACCGAAACGC GCGAGGCAGC	50
AAGCTTGGCA GAAATGGTTG AACTCCCGAG AGTGTCTTAC ACCTAGGGGA	100
GAAGCAGCCA AGGGGTTGTT TCCCACCAAG GACGACCCGT CTGCGCACAA	150
ACGGATGAGC CCATCAGACA AAGACATATT CATTCTCTGC TGCAAACATTG	200
GCATAGCTCT GCTTGGCTG GGGCTATTGG GGGAAAGTTGC GGTCGTGCT	250
CGCAGGGCTC TCACCCCTGA CTCTTCAAT AATAACTCTT CTGTGCAAGA	300
TTACAATCTA AACAAATTCTGG AGAACTCGAC CTTCCCTCCTG AGGCAAGGAC	350
CACAGCCAAC TTCCCTCTTAC AAGCCGCATC GATTTGTCC TTCAGAAATA	400
GAAATAAGAA TGCTTGCTAA AAATTATATT TTTACCAATA AGACCAATCC	450
AATAGGTAGA TTATTAGTTA CTATGTTAAG AAATGAATCA TTATCTTTA	500
GTACTATTT TACTCAAATT CAGAAGTTAG AAATGGGAAT AGAAAATAGA	550
AAGAGACGCT CAACCTCAAT TGAAGAACAG GTGCAAGGAC TATTGACCAC	600
AGGCCTAGAA GTAAAAAAGG GAAAAAAGAG TGTTTTGTC AAAATAGGAG	650
ACAGGTGGTG GCAACCAGGG ACTTATAGGG GACCTTACAT CTACAGACCA	700
ACAGATGCC CTTTACCAT A TACAGGAAGA TATGACTTAA ATTGGGATAG	750
GTGGGTTACA GTCAATGGCT ATAAAGTGT T ATATAGATCC CTCCCCTTTC	800
GTGAAAGACT CGCCAGAGCT AGACCTCCTT GGTGTATGTT GTCTCAAGAA	850
AAGAAAGACG ACATGAAACA ACAGGTACAT GATTATATT ATCTAGGAAC	900

AGGAATGCAC	TTTGCGGAA	AGATTTCCA	TACCAAGGAG	GGGACAGTGG	950
CTGGACTAAT	AGAACATTAT	TCTGCAAAAA	CTTATGGCAT	GAGTTATTAT	1000
GATTAGCCTT	GATTTGCCA	ACCTTGCAGT	TCCCAAGGCT	TAAGTAAGTT	1050
TTTGGTTACA	AACTGTTCTT	AAAACAAGGA	TGTGAGACAA	GTGGTTTCCT	1100
GACTTGGTTT	GGTATCAAAG	GTTCTGATCT	GAGCTCTGAG	TGTTCTATTT	1150
TCCTATGTTTC	TTTGGAATT	TATCCAAATC	TTATGTAAAT	GCTTATGTAA	1200
ACCAAGATAT	AAAAGAGTGC	TGATTTTTG	AGTAAACTTG	CAACAGTCCT	1250
AACATTCAACC	TCTTGTGTGT	TTGTGTCTGT	TCGCCATCCC	GTCTCCGCTC	1300
GTCACCTATC	CTTCACTTTC	CAGAGGGTCC	CCCCGCAGAC	CCCGCGGACC	1350
CTCAGGTCGG	CCGACTGCGG	CAGCTGGCGC	CCGAACAGGG	ACCCTCGGAT	1400
AAGTGACCCCT	TGTCTTTATT	TCTACTATTT	TGTGTTCGTC	TTGTTTGTC	1450
TCTATCTTGT	CTGGCTATCA	TCACAAGAGC	GGAACGGACT	CACCTCAGGG	1500
AACCAAGCTA	GCCCAATTG	ATGACTACGT	CCGGCGTTCC	ATTTGGCATG	1550
ACACTACGAC	CAACACGATC	TCGGTTGTCT	CGGCGCACTC	CGTACAGTAG	1600
GGATCGTCTA	CCTCCCTTTG	AGACAGAAAC	CCCGCGCTACC	ATACTGGAGG	1650
ATCATCCGCT	GCTGCCGAA	TGTAACACTT	TGACAATGCA	CAACGTGAGT	1700
TACGTGCGAG	GTCTCCCTG	CAGTGTGGGA	TTTACGCTGA	TTCAGGAATG	1750
GGTTGTTCCC	TGGGATATGG	TTCTAACGCG	GGAGGGAGCTT	GTAATCCTGA	1800
GGAAGTGTAT	GCACGTGTGC	CTGTGTTGTG	CCAACATTGA	TATCATGACG	1850
AGCATGATGA	TCCATGGTTA	CGAGTCCTGG	GCTCTCCACT	GTCATTGTTTC	1900
CAGTCCCGGT	TCCCTGCAGT	GTATAGCCGG	CGGGCAGGTT	TTGCCAGCT	1950
GGTTTAGGAT	GGTGGTGGAT	GGGCCATGT	TTAACAGAG	GTGTATATGG	2000
TACCGGGAGG	TGGTGAATTA	CAACATGCCA	AAAGAGGTAA	TGTTATGTC	2050
CAGCGTGT	ATGAGGGGTC	GCCACTTAAT	CTACCTGCGC	TTGTGGTATG	2100
ATGGCCACGT	GGGTTCTGTG	GTCCCCGCCA	TGAGCTTTGG	ATACAGCGCC	2150
TTGCACTGTG	GGATTTGAA	CAATATTGTG	GTGCTGTGCT	GCAGTTACTG	2200

TGCTGATT	AGTGAGATCA	GGGTGCGCTG	CTGTGCCCGG	AGGACAAGGC	2250
GCCTTATGCT	GCAGGGCGGTG	CGAACATCATCG	CTGAGGAGAC	CACTGCCATG	2300
TTGTATTCC	GCAGGGACGGA	GCAGGGCGCGG	CAGCAGTTA	TTCGCGCGCT	2350
GCTGCAGCAC	CACCGCCCTA	TCCTGATGCA	CGATTATGAC	TCTACCCCCA	2400
TGTAGGGATC	CAAGCTTGC	GGCGCATCGA	TGATATCAAG	CTTGCATGCC	2450
TGCAGGTCGA	CTCTAGAGGA	TCCCAGGGTGG	NATCCCTGTG	ACCCCTCCCC	2500
AGTGCCTCTC	CTGGCCCTGG	AAGTTGGCAC	TCCAGTGCC	ACCAGCCTTG	2550
TCCTAATAAA	ATTAAGTTGN	ATCATTGTTGT	CTGACTAGGT	GTCCTTCTAT	2600
AATATTATGG	GGTGGAGGGG	GGTGGTATGG	AGCAANGGN	AANTTGGNAA	2650
GACAANCTGT	AGGGCCTGCG	GGGTCTATTG	GGAACAAGCT	GGAGTGCAGT	2700
GGCACAAATCT	TGGCTCACTG	CAATCTCCGC	CTCCTGGGTT	CAAGCGATT	2750
TCCTGCCTCA	GAATCCCGAG	TTGTTGGGAT	TCCAGGCATG	CATGACCAGG	2800
CTCAGATAAT	TTTTGTTTTT	TTGGTAGAGA	CGGGGTTTCA	CCATATTGGN	2850
CAGGCTGGTC	TCCAACTCCT	AATCTCAGGT	GATCTNCCCA	CCTTGGCCTC	2900
CCAAATTGCT	GGGATTACAG	GNGTGAACCA	CTGNTCCCTT	CCCTGTCCTT	2950
CTGATTTAA	AATAACTATA	CCAGCAGGAG	GACGTCCAGA	CACAGCATAG	3000
GCTACCTGGC	CATGCCAAC	CGGTGGGACA	TTTGAGTTGC	TTGCTTGGCA	3050
CTGTCCCTCTC	ATGCCATTGGG	TCCACTCAGT	AGATGCCTGT	TGAATTGGGT	3100
ACGGGCCAG	CTTGGCTGTG	GAATGTGTGT	CAGTTAGGGT	GTGGAAAGTC	3150
CCCAGGCTCC	CCAGCAGGCA	GAAGTATGCA	AAGCATGCAT	CTCAATTAGT	3200
CAGCAACCAG	GTGTGGAAAG	TCCCCAGGCT	CCCCAGCAGG	CAGAAGTATG	3250
CAAAGCATGC	ATCTCAATT	GTCAGNAACC	ATAGNCCCGC	CCCTAACTCC	3300
GTCCATCCCG	GCCCTAACTC	NGGCCAGTTC	CGACCNTNCT	CCGGCNNATG	3350
GNTGAGTAAT	TTGCNNGATT	TATGCAGNNG	GCGAGGNCGC	CTCGGGCTCT	3400
GAGNTNTTCC	AGAAGTAGTG	AGGAGGCTTT	NNTGGTGGAA	TTGATCAGCT	3450
TGGGATCTGA	TCAAGAGACA	GGATGAGGAT	CGNNNCGNAT	GATTGAACAA	3500

72

GATGGGTTGC	ACGGAGGTTC	TCCGGNCGCT	TGGGTGGGGA	GGNTATTCGG	3550
NTATTNTTGG	TGNACAAACAG	NNAAACGGNT	GTTCTGATGC	CGCCGCGTTC	3600
NCGCTTCAG	NGCAGGGGGG	CCCCCCTCT	NTTGAGANNA	GCNCCCTTN	3650
TTG					3653

## (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 294 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Thr	Thr	Ser	Gly	Val	Pro	Phe	Gly	Met	Thr	Leu	Arg	Pro	Thr	Arg	
1					5				10					15		
Ser	Arg	Leu	Ser	Arg	Arg	Arg	Thr	Pro	Tyr	Ser	Arg	Asp	Arg	Leu	Pro	Pro
						20			25					30		
Phe	Glu	Thr	Glu	Thr	Arg	Ala	Thr	Ile	Leu	Glu	Asp	His	Pro	Leu	Leu	
						35			40				45			
Pro	Glu	Cys	Asn	Thr	Leu	Thr	Met	His	Asn	Val	Ser	Tyr	Val	Arg	Gly	
						50			55				60			
Leu	Pro	Cys	Ser	Val	Gly	Phe	Thr	Leu	Ile	Gln	Glu	Trp	Val	Val	Pro	
						65			70			75		80		
Trp	Asp	Met	Val	Leu	Thr	Arg	Glu	Glu	Leu	Val	Ile	Leu	Arg	Lys	Cys	
						85			90				95			
Met	His	Val	Cys	Leu	Cys	Cys	Ala	Asn	Ile	Asp	Ile	Met	Thr	Ser	Met	
						100			105				110			
Met	Ile	Tyr	Gly	Tyr	Glu	Ser	Trp	Ala	Leu	His	Cys	His	Cys	Ser	Ser	
						115			120				125			
Pro	Gly	Ser	Leu	Gln	Cys	Ile	Ala	Gly	Gly	Gln	Val	Leu	Ala	Ser	Trp	
						130			135			140				
Phe	Arg	Met	Val	Val	Asp	Gly	Ala	Met	Phe	Asn	Gln	Arg	Phe	Ile	Trp	
						145			150			155		160		

Tyr Arg Glu Val Val Asn Tyr Asn Met Pro Lys Glu Val Met Phe Met  
 165 170 175

Ser Ser Val Phe Met Arg Gly Arg His Leu Ile Tyr Leu Arg Tyr Trp  
 180 185 190

Tyr Asp Gly His Val Gly Ser Val Val Pro Ala Met Ser Phe Gly Tyr  
 195 200 205

~~Ser Ala Leu His Cys Gly Ile Leu Asn Asn Ile Val Val Leu Cys Cys~~  
 210 215 220

Ser Tyr Cys Ala Asp Leu Ser Glu Ile Arg Val Arg Cys Cys Ala Arg  
 225 230 235 240

Arg Thr Arg Arg Leu Met Leu Arg Ala Val Arg Ile Ile Ala Glu Glu  
 245 250 255

Thr Thr Ala Met Leu Tyr Ser Cys Arg Thr Glu Arg Arg Arg Gln Gln  
 260 265 270

Phe Ile Arg Ala Leu Leu Gln His His Arg Pro Ile Leu Met His Asp  
 275 280 285

Tyr Asp Ser Thr Pro Met  
 290

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35408 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATCATCAAT AATATAACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG	50
GGGGTGGAGT TTGTGACGTG GCGCGGGCG TGGGAACGGG GCGGGTGACG	100
TAGTAGTGTG GCGGAAGTGT GATGTTGCAA GTGTGGCGGA ACACATGTAA	150
GCGACGGATG TGGCAAAAGT GACGTTTTG GTGTGCGCCG GTGTACACAG	200
GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG TAAATTGGG	250
CGTAACCGAG TAAGATTGG CCATTTCGC GGGAAAACGT AATAAGAGGA	300

AGTGAAATCT	GAATAATTT	GTGTTACTCA	TAGCGCGTAA	TATTTGTCTA	350
GGGAGATCAG	CCTGCAGGTC	GTTACATAAC	TTACGGTAAA	TGGCCCGCCT	400
GGCTGACCGC	CCAACGACCC	CCGCCATTG	ACGTCAATAA	TGACGTATGT	450
TCCCCATAGTA	ACGCCAATAG	GGACTTTCCA	TTGACGTCAA	TGGGTGGAGT	500
ATTACGGTA	AACTGCCAC	TTGGCAGTAC	ATCAAGTGT	TCATATGCCA	550
AGTACGCC	CTATTGACGT	CAATGACGGT	AAATGGCCCG	CCTGGCATT	600
TGCCCAGTAC	ATGACCTTAT	GGGACTTTCC	TACTTGGCAG	TACATCTACT	650
CGAGGCCACG	TTCTGCTTCA	CTCTCCCCAT	CTCCCCCCCC	TCCCCACCCC	700
CAATTTGT	TTTATTTATT	TTTTAATTAT	TTTGTGCAGC	GATGGGGCG	750
GGGGGGGGGG	GGGGGCGCGC	GCCAGGCGGG	GCAGGGCGGG	GCGAGGGCG	800
GGCGGGGGCG	AGCGGGAGAG	GTGCGCGGC	AGCCAATCAG	AGCGCGCGC	850
TCCGAAAGTT	TCCTTTATG	GCGAGGCGGC	GGCGGCGGGC	GCCCTATAAA	900
AAGCGAAGCG	CGCGGCGGGC	GGGAGCGGG	TCAGCCACCG	CGGTGGCGGC	950
CGCAATTCCC	GGGGATCGAA	AGAGCCTGCT	AAAGCAAAA	AGAAGTCACC	1000
ATGTCGTTA	CTTGACCAA	CAAGAACGT	ATTTTCGTTG	CCGGTCTGGG	1050
AGGCATTGGT	CTGGACACCA	GCAAGGAGCT	GCTCAAGCGC	GATCCCGTCG	1100
TTTACAACG	TCGTGACTGG	GAAAACCTG	GCGTTACCCA	ACTTAATCGC	1150
CTTGCAGCAC	ATCCCCCTT	CGCCAGCTGG	CGTAATAGCG	AAGAGGCCG	1200
CACCGATCGC	CCTTCCCAAC	AGTTGCCAG	CCTGAATGGC	GAATGGCGCT	1250
TTGCCTGGTT	TCCGGCACCA	GAAGCGGTGC	CGGAAAGCTG	GCTGGAGTGC	1300
GATCTTCCTG	AGGCCGATAC	TGTCGTCGTC	CCCTCAAAC	GGCAGATGCA	1350
CGGTTACGAT	GCGCCCATCT	ACACCAACGT	AACCTATCCC	ATTACGGTCA	1400
ATCCGCCGTT	TGTTCCCACG	GAGAACCGA	CGGGTTGTTA	CTCGCTCACA	1450
TTTAATGTTG	ATGAAAGCTG	GCTACAGGAA	GGCCAGACGC	GAATTATTT	1500
TGATGGCGTT	AACTCGGC	TTCATCTGTG	GTGCAACGGG	CGCTGGGT	1550
GTTACGGCCA	GGACAGTCGT	TTGCCGTCTG	AATTTGACCT	GAGCGCATT	1600

TTACCGCGCCG	GAGAAAACCG	CCTCGCGGTG	ATGGTGCTGC	GTTGGAGTGA	1650
CGGCAGTTAT	CTGGAAGATC	AGGATATGTG	GCGGATGAGC	GGCATTTC	1700
GTGACGTCTC	GTTGCTGCAT	AAACCGACTA	CACAAATCAG	CGATTTCCAT	1750
GTTGCCACTC	GCTTTAATGA	TGATTCAGC	CGCGCTGTAC	TGGAGGCTGA	1800
AGTTCA	GATG	TGCGGCGAGT	TGCGTGACTA	CCTACGGGT	1850
TATGGCAGGG	TGAAACCGAG	GTCGCCAGCG	GCACCGCGCC	TTTCGGCGGT	1900
GAAATTATCG	ATGAGCGTGG	TGGTTATGCC	GATCGCGTCA	CACTACGTCT	1950
GAACGTCGAA	AACCCGAAAC	TGTGGAGCGC	CGAAATCCCG	AATCTCTATC	2000
GTGCGGTGGT	TGAAC	TGAC	ACCGCCGACG	GCACGCTGAT	2050
GCCTGCGATG	TCGGTTTCCG	CGAGGTGCGG	ATTGAAAATG	GTCTGCTGCT	2100
GCTGAACGGC	AAGCCGTTGC	TGATTGAGG	CGTTAACCGT	CACGAGC	2150
ATCCTCTGCA	TGGTCAGGTC	ATGGATGAGC	AGACGATGGT	GCAGGGATATC	2200
CTGCTGATGA	AGCAGAACAA	CTTTAACGCC	GTGCGCTGTT	CGCATTATCC	2250
GAACCATCCG	CTGTGGTACA	CGCTGTGCGA	CCGCTACGGC	CTGTATGTGG	2300
TGGATGAAGC	CAATATTGAA	ACCCACGGCA	TGGTGCCAAT	GAATCGTCTG	2350
ACCGATGATC	CGCGCTGGCT	ACCGGCGATG	AGCGAACGCG	TAACCGAAT	2400
GGTGCAGCGC	GATCGTAATC	ACCCGAGTGT	GATCATCTGG	TCGCTGGGGA	2450
ATGAATCAGG	CCACGGCGCT	AATCACGACG	CGCTGTATCG	CTGGATCAAA	2500
TCTGTCGATC	CTTCCCGCCC	GGTGCAGTAT	GAAGGCGGCG	GAGCCGACAC	2550
CACGGCCACC	GATATTATTT	GCCCGATGTA	CGCGCGCGTG	GATGAAGACC	2600
AGCCCTTCCC	GGCTGTGCCG	AAATGGTCCA	TCAAAAAATG	GCTTTCGCTA	2650
CCTGGAGAGA	CGCGCCCGCT	GATCCTTGC	GAATACGCC	ACGCGATGGG	2700
TAACAGTCTT	GGCGGTTTCG	CTAAATACTG	GCAGGC	CGTCAGTATC	2750
CCCGTTACA	GGGCGGCTTC	GTCTGGACT	GGGTGGATCA	GTGCGCTGATT	2800
AAATATGATG	AAAACGGCAA	CCC GTGGTCG	GCTTACGGCG	GTGATTTGG	2850
CGATACGCCG	AACGATCGCC	AGTTCTGTAT	GAACGGTCTG	GTCTTGCCG	2900

ACCGCACGCC	GCATCCAGCG	CTGACGGAAG	CAAAACACCA	GCAGCAGTTT	2950
TTCCAGTTCC	TTTTATCCGG	GCAAACCATC	GAAGTGACCA	GCGAATACCT	3000
GTTCCGTCAT	AGCGATAACG	AGCTCCTGCA	CTGGATGGTG	GCGCTGGATG	3050
GTAAGCCGCT	GGCAAGCGGT	GAAGTGCCCTC	TGGATGTCCG	TCCACAAGGT	3100
AAACAGTTGA	TTGAAC TGCC	TGAAC TACCG	CAGCCGGAGA	GCGCCGGGCA	3150
ACTCTGGCTC	ACAGTACGCG	TAGTGCAACC	GAACGCGACC	GCATGGTCAG	3200
AAGCCGGGCA	CATCAGCGCC	TGGCAGCAGT	GGCGTCTGGC	GGAAAACCTC	3250
AGTGTGACGC	TCCCCGCCGC	GTCCCACGCC	ATCCCGCATC	TGACCAACCAG	3300
CGAAATGGAT	TTTTGCATCG	AGCTGGGTAA	TAAGCGTTGG	CAATTAAACC	3350
GCCAGTCAGG	CTTTCTTC	CAGATGTGGA	TTGGCGATAAA	AAAACAAC	3400
CTGACGCCGC	TGCGCGATCA	GTTCACCCGT	GCACCGCTGG	ATAACGACAT	3450
TGGCGTAAGT	GAAGCGACCC	GCATTGACCC	TAACGCCCTGG	GTCGAACGCT	3500
GGAAGGCAGGC	GGGCCATTAC	CAGGCCGAAG	CAGCGTTGTT	GCAGTGCACG	3550
GCAGATACAC	TTGCTGATGC	GGTGCTGATT	ACGACCGCTC	ACGCGTGGCA	3600
GCATCAGGGG	AAAACCTTAT	TTATCAGCCG	GAAAACCTAC	CGGATTGATG	3650
GTAGTGGTCA	AATGGCGATT	ACCGTTGATG	TTGAAGTGGC	GAGCGATA	3700
CCGCATCCGG	CGCGGATTGG	CCTGAAC	CAGCTGGCGC	AGGTAGCAGA	3750
GCGGGTAAAC	TGGCTCGGAT	TAGGGCCGCA	AGAAAAC	CCCGACCGCC	3800
TTACTGCCGC	CTGTTTGAC	CGCTGGGATC	TGCCATTGTC	AGACATGTAT	3850
ACCCCGTACG	TCTTCCCGAG	CGAAAACGGT	CTGCGCTGCG	GGACCGCGA	3900
ATTGAATTAT	GGCCCACACC	AGTGGCGCGG	CGACTTCCAG	TTCAACATCA	3950
GCCGCTACAG	TCAACAGCAA	CTGATGGAAA	CCAGCCATCG	CCATCTGCTG	4000
CACGCCGAAG	AAGGCACATG	GCTGAATATC	GACGGTTTCC	ATATGGGAT	4050
TGGTGGCGAC	GA	CTGCTGGGA	GCCC GTCA	GT	4100
GCGCCGGTCG	CTACCATTAC	CAGTTGGTCT	GGTGTCAAAA	ATAATAATAA	4150
CCGGGCAGGC	CATGTCTGCC	CGTATTCG	GTAAGGAAAT	CCATTATGTA	4200

CTATTTAAAA AACACAAACT TTTGGATGTT CGGTTTATTTC TTTTTCTTTT	4250
ACTTTTTAT CATGGGAGCC TACTTCCCCT TTTTCCCGAT TTGGCTACAT	4300
GACATCAACC ATATCAGCAA AAGTGATACG GGTATTATTT TTGCCGCTAT	4350
TTCTCTGTTT TCGCTATTAT TCCAACCGCT GTTTGGTCTG CTTTCTGACA	4400
AACTCGGCCT CGACTCTAGG CGGCCGCGGG GATCCAGACA TGATAAGATA	4450
CATTGATGAG TTTGGACAAA CCACAACCTAG AATGCAGTGA AAAAAATGCT	4500
TTATTTGTGA AATTGTGAT GCTATTGCTT TATTGTAAAC CATTATAAGC	4550
TGCAATAAAC AAGTTAACAA CAACAATTGC ATTCACTTTA TGTTTCAGGT	4600
TCAGGGGGAG GTGTGGGAGG TTTTTCCGA TCCTCTAGAG TCGACCTGCA	4650
GGCTGATCAG TGGAAGGTGC TGAGGTACGA TGAGACCCGC ACCAGGTGCA	4700
GACCCCTGCCA GTGTGGCGGT AAACATATTA GGAACCAGCC TGTGATGCTG	4750
GATGTGACCG AGGAGCTGAG GCCCGATCAC TTGGTGCTGG CCTGCACCCG	4800
CGCTGAGTTT GGCTCTAGCG ATGAAGATAAC AGATTGAGGT ACTGAAATGT	4850
GTGGGCGTGG CTTAAGGGTG GGAAAGAATA TATAAGGTGG GGGTCTTATG	4900
TAGTTTGTA TCTGTTTGC AGCAGCCGCC GCCGCCATGA GCACCAAATC	4950
GTTTGATGGA AGCATTGTGA GCTCATATTT GACAACGCCG ATGCCCCCAT	5000
GGGCCGGGGT GCGTCAGAAT GTGATGGCT CCAGCATTGA TGGTCGCC	5050
GTCCTGCCCG CAAACTCTAC TACCTTGACC TACGAGACCG TGTCTGGAAC	5100
GCCGTTGGAG ACTGCAGCCT CCGCCGCCGC TTCAGCCGCT GCAGCCACCG	5150
CCCGCGGGAT TGTGACTGAC TTTGCTTCC TGAGCCCGCT TGCAAGCAGT	5200
GCAGCTTCCC GTTCATCCGC CCGCGATGAC AAGTTGACGG CTCTTTGGC	5250
ACAATTGGAT TCTTGACCC GGGAACTTAA TGTCGTTCT CAGCAGCTGT	5300
TGGATCTGCG CCAGCAGGTT TCTGCCCTGA AGGCTCCCTC CCCTCCCAAT	5350
GCGGTTAAA ACATAAATAA AAAACCAGAC TCTGTTGGA TTTGGATCAA	5400
GCAAGTGTCT TGCTGTCTTT ATTTAGGGGT TTTGCGCGCG CGGTAGGCC	5450
GGGACCAGCG GTCTCGGTG TTGAGGGTCC TGTGTATTT TTCCAGGACG	5500

TGGTAAAGGT	GACTCTGGAT	GTTCAGATAAC	ATGGGCATAA	GCCCGTCTCT	5550
GGGGTGGAGG	TAGCACCAC	GCAGAGCTTC	ATGCTGCGGG	GTGGTGTGTTGT	5600
AGATGATCCA	GTCGTAGCAG	GAGCGCTGGG	CGTGGTGCCT	AAAAATGTCT	5650
TTCAGTAGCA	AGCTGATTGC	CAGGGGCAGG	CCCTTGGTGT	AAGTGTTCAC	5700
AAAGCGGTTA	AGCTGGGATG	GGTGCATAACG	TGGGGATATG	AGATGCATCT	5750
TGGACTGTAT	TTTTAGGTTG	GCTATGTTCC	CAGCCATATC	CCTCCGGGGA	5800
TTCATGTTGT	GCAGAACAC	CAGCACAGTG	TATCCGGTGC	ACTTGGGAAA	5850
TTTGTATGT	AGCTTAGAAG	GAAATGCGTG	GAAGAACTTG	GAGACGCCCT	5900
TGTGACCTCC	AAGATTTCC	ATGCATTCTG	CCATAATGAT	GGCAATGGGC	5950
CCACGGGCGG	CGGCCTGGC	GAAGATATT	CTGGGATCAC	TAACGTCATA	6000
GTGTGTTCC	AGGATGAGAT	CGTCATAGGC	CATTTTACA	AAGCGCGGGC	6050
GGAGGGTGCC	AGACTGCGGT	ATAATGGTTC	CATCCGGCCC	AGGGGCGTAG	6100
TTACCCCTCAC	AGATTGCA	TTCCCCACGCT	TTGAGTTCA	ATGGGGGGAT	6150
CATGTCTACC	TGCGGGCGA	TGAAGAAAAC	GGTTTCCGGG	GTAGGGGAGA	6200
TCAGCTGGGA	AGAAAGCAGG	TTCCCTGAGCA	GCTGCGACTT	ACCGCAGCCG	6250
GTGGGCCCGT	AAATCACACC	TATTACCGGG	TGCAACTGGT	ACTTAAGAGA	6300
GCTGCAGCTG	CCGTCATCCC	TGAGCAGGGG	GGCCACTTCG	TTAAGCATGT	6350
CCCTGACTCG	CATGTTTCC	CTGACCAAAT	CCGCCAGAAG	GCGCTCGCCG	6400
CCCAGCGATA	GCAGTTCTTG	CAAGGAAGCA	AAGTTTTCA	ACGGTTTGAG	6450
ACCGTCCGCC	GTAGGCATGC	TTTGAGCGT	TTGACCAAGC	AGTCCAGGC	6500
GGTCCCACAG	CTCGGTCA	TGCTCTACGG	CATCTCGATC	CAGCATATCT	6550
CCTCGTTCG	CGGGTTGGGG	CGGCTTCGC	TGTACGGCAG	TAGTCGGTGC	6600
TCGTCCAGAC	GGGCCAGGGT	CATGTCTTC	CACGGGCGCA	GGGTCCCTCGT	6650
CAGCGTAGTC	TGGGTCACGG	TGAAGGGGTG	CGCTCCGGGC	TGCGCGCTGG	6700
CCAGGGTGCC	CTTGAGGCTG	GTCCTGCTGG	TGCTGAAGCG	CTGCCGGTCT	6750
TCGCCCTGCC	CGTCGGCCAG	GTAGCATTG	ACCATGGTGT	CATAGTCCAG	6800

CCCTCCGCG	CGTGGCCCT	TGGCGCGAG	CTTGCCTTG	GAGGAGGC	6850
CGCACGAGGG	GCAGTGCAGA	CTTTGAGGG	CGTAGAGCTT	GGCGCGAGA	6900
AATACCGATT	CCGGGGAGTA	GGCATCCGCG	CCGCAGGCC	CGCAGACGGT	6950
CTCGCATTCC	ACGAGCCAGG	TGAGCTCTGG	CCGTTGGGG	TCAAAACCA	7000
GGTTTCCCCC	ATGCTTTTG	ATGCCTTCT	TACCTCTGGT	TTCCATGAGC	7050
CGGTGTCCAC	GCTCGGTGAC	GAAAAGGCTG	TCCGTGTCCC	CGTATACAGA	7100
CTTGAGAGGC	CTGTCCTCGA	GCGGTGTTCC	GCGGTCTCC	TCGTATAGAA	7150
ACTCGGACCA	CTCTGAGACA	AAGGCTCGCG	TCCAGGCCAG	CACGAAGGAG	7200
GCTAAGTGGG	AGGGGTAGCG	GTCGTTGTCC	ACTAGGGGGT	CCACTCGCTC	7250
CAGGGTGTGA	AGACACATGT	CGCCCTCTTC	GGCATCAAGG	AAGGTGATTG	7300
GTTTGTAGGT	GTAAGGCCACG	TGACCGGGTG	TTCCTGAAGG	GGGGCTATAA	7350
AAGGGGGTGG	GGGCGCGTTC	GTCCTCACTC	TCTTCCGCAT	CGCTGTCTGC	7400
GAGGGCCAGC	TGTTGGGTG	AGTACTCCCT	CTGAAAAGCG	GGCATGACTT	7450
CTGCGCTAAG	ATTGTCAGTT	TCCAAAAACG	AGGAGGATT	GATATTCA	7500
TGGCCCGCGG	TGATGCCCTT	GAGGGTGGCC	GCATCCATCT	GGTCAGAAAA	7550
GACAATCTTT	TTGTTGTCAA	GCTTGGTGGC	AAACGACCCG	TAGAGGGCGT	7600
TGGACAGCAA	CTTGGCGATG	GAGCGCAGGG	TTTGGTTTTT	CTCGCGATCG	7650
GCGCGCTCCT	TGGCCCGCAT	GTTTAGCTGC	ACGTATTCGC	GCGCAACGCA	7700
CCGCCATTGCG	GGAAAGACGG	TGGTGCCTC	GTCGGGCACC	AGGTGCACGC	7750
GCCAACCGCG	GTTGTGCAGG	GTGACAAGGT	CAACGCTGGT	GGCTACCTCT	7800
CCCGCGTAGGC	GCTCGTTGGT	CCAGCAGAGG	CGGCCGCC	TGCGCGAGCA	7850
GAATGGCGGT	AGGGGGTCTA	GCTGCGTCTC	GTCCGGGGGG	TCTGCGTCCA	7900
CGGTAAAGAC	CCCGGGCAGC	AGGCGCGCGT	CGAAGTAGTC	TATCTTGCAT	7950
CCTTGCAAGT	CTAGCGCCTG	CTGCCATGCG	CGGGCGGCAA	GCGCGCGCTC	8000
GTATGGGTTG	AGTGGGGGAC	CCCATGGCAT	GGGGTGGGTG	AGCGCGGAGG	8050
CGTACATGCC	GCAAATGTG	TAAACGTAGA	GGGGCTCTCT	GAGTATTCCA	8100

80

AGATATGTAG GGTAGCATCT TCCACCGCGG ATGCTGGCGC GCACGTAATC	8150
GTATAGTTCG TGCGAGGGAG CGAGGAGGTC GGGACCGAGG TTGCTACGGG	8200
CGGGCTGCTC TGCTCGGAAG ACTATCTGCC TGAAGATGGC ATGTGAGTTG	8250
GATGATATGG TTGGACGCTG GAAGACGTTG AAGCTGGCGT CTGTGAGACC	8300
TACCGCGTCA CGCACGAAGG AGGCGTAGGA GTCGCGCAGC TTGTTGACCA	8350
GCTCGGCGGT GACCTGCACG TCTAGGGCGC AGTAGTCCAG GGTTTCCTTG	8400
ATGATGTCAT ACTTATCCTG TCCCTTTTT TTCCACAGCT CGCGGTTGAG	8450
GACAAACTCT TCGCGGTCTT TCCAGTACTC TTGGATCGGA AACCCGTCGG	8500
CCTCCGAACG GTAAGAGCCT AGCATGTAGA ACTGGTTGAC GGCCTGGTAG	8550
GCGCAGCATIC CCTTTTCTAC GGGTAGCGCG TATGCCTGCG CGGCCTTCCG	8600
GAGCGAGGTG TGGGTGAGCG CAAAGGTGTC CCTGACCATG ACTTTGAGGT	8650
ACTGGTATTG GAAGTCAGTG TCGTCGCATC CGCCCTGCTC CCAGAGCAAA	8700
AAAGTCCGTGC GCTTTTGGA ACGCGGATTT GGCAGGGCGA AGGTGACATC	8750
GTTGAAGAGT ATCTTCCCG CGCGAGGCAT AAAGTTGCGT GTGATGCGGA	8800
AGGGTCCCAGG CACCTCGGAA CGGTTGTTAA TTACCTGGGC GGCGAGCACG	8850
ATCTCGTCAA AGCCGTTGAT GTTGTGGCCC ACAATGTAAA GTTCCAAGAA	8900
GCGCGGGATG CCCTTGATGG AAGGCAATT TTTAAGTTCC TCGTAGGTGA	8950
GCTCTTCAGG GGAGCTGAGC CCGTGCTCTG AAAGGGCCCA GTCTGCAAGA	9000
TGAGGGTTGG AAGCGACGAA TGAGCTCCAC AGGTACCGGG CCATTAGCAT	9050
TTGCAGGTGG TCGCGAAAGG TCCTAAACTG GCGACCTATG GCCATTTTT	9100
CTGGGGTGAT GCAGTACAAG GTAAGCGGGT CTTGTTCCCA GCGGTCCCAT	9150
CCAAGGTTCG CGGCTAGGTC TCGCGCGGCA GTCACTAGAG GCTCATCTCC	9200
GCCGAACCTTC ATGACCAGCA TGAAGGGCAC GAGCTGCTTC CCAAAGGCC	9250
CCATCCAAGT ATAGGTCTCT ACATCGTAGG TGACAAAGAG ACGCTCGGTG	9300
CGAGGATGCG AGCCGATCGG GAAGAACTGG ATCTCCGCC ACCAATTGGA	9350
GGAGTGGCTA TTGATGTGGT GAAAGTAGAA GTCCCTGCGA CGGGCCGAAC	9400

ACTCGTGCTG	GCTTTGTAA	AAACGTGCGC	AGTACTGGCA	GCGGTGCACG	9450
GGCTGTACAT	CCTGCACGAG	GTTGACCTGA	CGACCGCGCA	CAAGGAAGCA	9500
GAGTGGGAAT	TTGAGCCCC	CGCCTGGCGG	GTTGGCTGG	TGGTCTTCTA	9550
CTTCGGCTGC	TTGTCCCTTGA	CCGTCTGGCT	GCTCGAGGGG	AGTTACGGTG	9600
GATCGGACCA	CCACGCCGCG	CGAGCCAAA	GTCCAGATGT	CCGCGCGCGG	9650
CGGTCGGAGC	TTGATGACAA	CATCGCGCAG	ATGGGAGCTG	TCCATGGTCT	9700
GGAGCTCCCG	CGGCGTCAGG	TCAGGGCGGA	GCTCCTGCAG	GTTTACCTCG	9750
CATAGACGGG	TCAGGGCGCG	GGCTAGATCC	AGGTGATAACC	TAATTTCCAG	9800
GGGCTGGTTG	GTGGCGCGT	CGATGGCTTG	CAAGAGGCCG	CATCCCCGCG	9850
GCGCGACTAC	GGTACCGCGC	GGCGGGCGGT	GGGCCGCGGG	GGTGTCCCTG	9900
GATGATGCAT	CTAAAAGCGG	TGACGGGGGC	GAGCCCCCGG	AGGTAGGGGG	9950
GGCTCCGGAC	CCGCCGGGAG	AGGGGGCAGG	GGCACGTCGG	CGCCGCGCGC	10000
GGGCAGGAGC	TGGTGCTGCG	CGCGTAGGTT	GCTGGCGAAC	GCGACGACGC	10050
GGCGGTTGAT	CTCCTGAATC	TGGCGCCTCT	GCGTGAAGAC	GACGGGCCCC	10100
GTGAGCTTGA	GCCTGAAAGA	GAGTCGACA	GAATCAATT	CGGTGTCGTT	10150
GACGGCGGCC	TGGCGAAAAA	TCTCCTGCAC	GTCTCCTGAG	TTGTCTTGAT	10200
AGGCGATCTC	GGCCATGAAC	TGCTCGATCT	CTTCCTCCTG	GAGATCTCCG	10250
CGTCCGGCTC	GCTCCACGGT	GGCGGCGAGG	TCGTTGGAAA	TGCGGGCCAT	10300
GAGCTGCGAG	AAGGCATTGA	GGCCTCCCTC	GTTCCAGACG	CGGCTGTAGA	10350
CCACGCCCCC	TTCGGCATCG	CGGGCGCGCA	TGACCACCTG	CGCGAGATTG	10400
AGCTCCACGT	GCCGGGCGAA	GACGGCGTAG	TTTCGCAGGC	GCTGAAAGAG	10450
GTAGTTGAGG	GTGGTGGCGG	TGTGTTCTGC	CACGAAGAAG	TACATAACCC	10500
AGCGTCGCAA	CGTGGATTCG	TTGATATCCC	CCAAGGCCTC	AAGGCGCTCC	10550
ATGGCCTCGT	AGAAGTCCAC	GGCGAAGTTG	AAAAACTGGG	AGTTGCGCGC	10600
CGACACGGTT	AACTCCTCCT	CCAGAAGACG	GATGAGCTCG	GCGACAGTGT	10650
CGCGCACCTC	GCGCTCAAAG	GCTACAGGGG	CCTCTTCTTC	TTCTTCAATC	10700

TCCTCTTCCA	TAAGGGCCTC	CCCTTCTTCT	TCTTCTGGCG	GCGGTGGGG	10750
AGGGGGGACA	CGGCGGCGAC	GACGGCGCAC	CGGGAGGCAG	TCGACAAAGC	10800
GCTCGATCAT	CTCCCCGCGG	CGACGGCGCA	TGGTCTCGGT	GACGGCGCGG	10850
CCGTTCTCGC	GGGGGCGCAG	TTGGAAGACG	CCGCCCCTCA	TGTCCCGGTT	10900
ATGGGTTGGC	GGGGGGCTGC	CATGCGGCAG	GGATACGGCG	CTAACGATGC	10950
ATCTCAACAA	TTGTTGTGTA	GGTACTCCGC	CGCCGAGGGA	CCTGAGCGAG	11000
TCCGCATCGA	CCGGATCGGA	AAACCTCTCG	AGAAAGGCAGT	CTAACCGAGTC	11050
ACAGTCGCAA	GGTAGGCTGA	GCACCGTGGC	GGGCGGCAGC	GGGCGGCGGT	11100
CGGGGTTGTT	TCTGGCGGAG	GTGCTGCTGA	TGATGTAATT	AAAGTAGGCG	11150
GTCTTGAGAC	GGCGGATGGT	CGACAGAAGC	ACCATGTCCT	TGGGTCCGGC	11200
CTGCTGAATG	CGCAGGCGGT	CGGCCATGCC	CCAGGCTTCG	TTTGACATC	11250
GGCGCAGGTC	TTTGTAGTAG	TCTTGCATGA	GCCTTTCTAC	CGGCACTTCT	11300
TCTTCTCCTT	CCTCTTGTCC	TGCATCTCTT	GCATCTATCG	CTGGGGCGGC	11350
GGCGGAGTTT	GGCCGTAGGT	GGCGCCCTCT	TCCTCCCATG	CGTGTGACCC	11400
CGAAAGCCCCT	CATCGGCTGA	AGCAGGGCTA	GGTCGGCGAC	AACCGCGCTCG	11450
GCTAATATGG	CCTGCTGCAC	CTGCGTGAGG	GTAGACTGGA	AGTCATCCAT	11500
GTCCACAAAG	CGGTGGTATG	CGCCCGTGT	GATGGTGTAA	GTGCAGTTGG	11550
CCATAACCGA	CCAGTTAACG	GTCTGGTGAC	CCGGCTGCCA	GAGCTCGGTG	11600
TACCTGAGAC	GCGAGTAAGC	CCTCGAGTCA	AATACGTAGT	CGTTGCAAGT	11650
CCGCACCAGG	TACTGGTATC	CCACCAAAAA	GTGCGGCGGC	GGCTGGCGGT	11700
AGAGGGGCCA	GCGTAGGGTG	GCCGGGGCTC	CGGGGGCGAG	ATCTTCAAAC	11750
ATAAGGCGAT	GATATCCGTA	GATGTACCTG	GACATCCAGG	TGATGCCGGC	11800
GGCGGTGGTG	GAGGCGCGCG	GAAAGTCGCG	GACGCGGTTG	CAGATGTTGC	11850
GCAGCGGCAA	AAAGTGCTCC	ATGGTCGGGA	CGCTCTGGCC	GGTCAGGCGC	11900
GCGCAATCGT	TGACGCTCTA	GACCGTGCAA	AAGGAGAGCC	TGTAAGCGGG	11950
CACTCTTCCG	TGGTCTGGTG	GATAAATTG	CAAGGGTATC	ATGGCGGACG	12000

ACCGGGGTTTC	GAGCCCCGTA	TCCGGCCGTC	CGCCGTGATC	CATGCGGTTA	12050
CCGCCCCGCGT	GTCGAACCCA	GGTGTGCGAC	GTCAGACAAAC	GGGGGAGTGC	12100
TCCTTTGGC	TTCCTTCCAG	GCGCGGCCGC	TGCTGCGCTA	GCTTTTTGG	12150
CCACTGGCCG	CGCGCAGCGT	AAGCGGTTAG	GCTGGAAAGC	GAAAGCATT	12200
AGTGGCTCGC	TCCCTGTAGC	CGGAGGGTTA	TTTTCCAAGG	GTTGAGTCGC	12250
GGGACCCCCCG	GTTCGAGTCT	CGGACCGGCC	GGACTGCGGC	GAACGGGGGT	12300
TTGCCTCCCC	GTCATGCAAG	ACCCCGCTTG	CAAATTCCCTC	CGGAAACAGG	12350
GACGAGCCCC	TTTTTGCTT	TTCCCAGATG	CATCCGGTGC	TGCGGCAGAT	12400
GCGCCCCCCT	CCTCAGCAGC	GGCAAGAGCA	AGAGCAGCGG	CAGACATGCA	12450
GGGCACCCCTC	CCCTCCTCCT	ACCGCGTCAG	GAGGGGCGAC	ATCCGCGGTT	12500
GACCGGGCAG	CAGATGGTGA	TTACGAACCC	CCGGGGCGCC	GGGCCCCGGCA	12550
CTACCTGGAC	TTGGAGGAGG	GCGAGGGCCT	GGCGCGGCTA	GGAGCGCCCT	12600
CTCCTGAGCG	GTACCCAAGG	GTGCAGCTGA	AGCGTGTATAC	GCGTGAGGCG	12650
TACGTGCCGC	GGCAGAACCT	GTTCGCGAC	CGCGAGGGAG	AGGAGCCCGA	12700
GGAGATGCGG	GATCGAAAGT	TCCACGCAGG	GCGCGAGCTG	CGGCATGGCC	12750
TGAATCGCGA	GCGGTTGCTG	CGCGAGGAGG	ACTTTGAGCC	CGACGCGCGA	12800
ACCGGGATTA	GTCCCGCGCG	CGCACACGTG	GCGGCCGCG	ACCTGGTAAC	12850
CGCATACGAG	CAGACGGTGA	ACCAGGAGAT	TAACTTCAA	AAAAGCTTTA	12900
ACAACCACGT	GCGTACGCTT	GTGGCGCGCG	AGGAGGTGGC	TATAGGACTG	12950
ATGCATCTGT	GGGACTTTGT	AAGCGCGCTG	GAGCAAAACC	CAAATAGCAA	13000
GCCGCTCATG	GCGCAGCTGT	TCCTTATAGT	GCAGCACAGC	AGGGACAACG	13050
AGGCATTCAAG	GGATGCGCTG	CTAACACATAG	TAGAGCCCCA	GGGCCGCTGG	13100
CTGCTCGATT	TGATAAACAT	CCTGCAGAGC	ATAGTGGTGC	AGGAGCGCAG	13150
CTTGAGCCTG	GCTGACAAGG	TGGCCGCCAT	CAACTATTCC	ATGCTTAGCC	13200
TGGGCAAGTT	TTACGCCCGC	AAGATATAACC	ATACCCCTTA	CGTTCCCATA	13250
GACAAGGAGG	TAAAGATCGA	GGGGTTCTAC	ATGCGCATGG	CGCTGAAGGT	13300

GCTTACCTTG AGCGACGACC TGGGCGTTA TCGCAACGAG CGCATCCACA 13350  
AGGCCGTGAG CGTGAGCCGG CGGCGCGAGC TCAGCGACCG CGAGCTGATG 13400  
CACAGCCTGC AAAGGGCCCT GGCTGGCACG GGCAGCGGCG ATAGAGAGGC 13450  
CGAGTCCTAC TTTGACGCGG GCGCTGACCT GCGCTGGGCC CCAAGCCGAC 13500  
GCGCCCTGGA GGCAGCTGGG GCCGGACCTG GGCTGGCGGT GGCACCCGCG 13550  
CGCGCTGGCA ACGTCGGCGG CGTGGAGGAA TATGACGAGG ACGATGAGTA 13600  
CGAGCCAGAG GACGGCGAGT ACTAAGCGGT GATGTTCTG ATCAGATGAT 13650  
GCAAGACGCA ACGGACCCGG CGGTGCGGGC GGCGCTGCAG AGCCAGCCGT 13700  
CCGGCCTTAA CTCCACGGAC GACTGGCGCC AGGTCAATGGA CCGCATCATG 13750  
TCGCTGACTG CGCGCAATCC TGACGCGTTC CGGCAGCAGC CGCAGGCCAA 13800  
CCGGCTCTCC GCAATTCTGG AAGCGGTGGT CCCGGCGCGC GCAAACCCCA 13850  
CGCACGAGAA GGTGCTGGCG ATCGTAAACG CGCTGGCCGA AAACAGGGCC 13900  
ATCCGGCCCG ACGAGGCCGG CCTGGTCTAC GACGCGCTGC TTCAGCGCGT 13950  
GGCTCGTTAC AACAGCGGCA ACGTGCAGAC CAACCTGGAC CGGCTGGTGG 14000  
GGGATGTGCG CGAGGCCGTG GCGCAGCGTG AGCGCGCGCA GCAGCAGGGC 14050  
AACCTGGGCT CCATGGTGC ACTAAACGCC TTCCTGAGTA CACAGCCCGC 14100  
CAACGTGCCG CGGGGACAGG AGGACTACAC CAACTTTGTG AGCCCACTGC 14150  
GGCTAATGGT GACTGAGACA CCGCAAAGTG AGGTGTACCA GTCTGGGCCA 14200  
GACTATTTT TCCAGACCAG TAGACAAGGC CTGCAGACCG TAAACCTGAG 14250  
CCAGGCTTTC AAAAACTTGC AGGGGCTGTG GGGGGTGCAG GCTCCCACAG 14300  
GCGACCGCGC GACCGTGTCT AGCTTGCTGA CGCCCAACTC GCGCCTGTTG 14350  
CTGCTGCTAA TAGGCCCTT CACGGACAGT GGCAGCGTGT CCCGGGACAC 14400  
ATACCTAGGT CACTTGCTGA CACTGTACCG CGAGGCCATA GGTCAGGCC 14450  
ATGTGGACGA GCATACTTTC CAGGAGATTA CAAGTGTCAAG CCGCGCGCTG 14500  
GGGCAGGAGG ACACGGGCAG CCTGGAGGCA ACCCTAAACT ACCTGCTGAC 14550  
CAACCAGCGG CAGAAGATCC CCTCGTTGCA CAGTTAAAC AGCGAGGAGG 14600

AGCGCATTTC GCGCTACGTG CAGCAGAGCG TGAGCCTTAA CCTGATGCAGC 14650  
GACGGGGTAA CGCCCAGCGT GGCGCTGGAC ATGACCGCGC GCAACATGGA 14700  
ACCGGGCATG TATGCCTCAA ACCGGCCGTT TATCAACCAGC CTAATGGACT 14750  
ACTTGCATCG CGCGGCCGCC GTGAACCCCG AGTATTCAC CAATGCCATC 14800  
TTGAACCCGC ACTGGCTACC GCCCCCTGGT TTCTACACCG GGGGATTCGA 14850  
GGTGGCCCGAG GGTAACGATG GATTCCCTCG GGACGACATA GACCGACAGCG 14900  
TGTGTTCCCC GCAACCGCAG ACCCTGCTAG AGTTGCAACA GCGCGAGCAG 14950  
GCAGAGGCCGG CGCTGCGAAA GGAAAGCTTC CGCAGGCCAA GCAGCTTGTC 15000  
CGATCTAGGC GCTGCGGCCCG CGCGGTCAAGA TGCTAGTAGC CCATTCCAA 15050  
GCTTGATAGG GTCTCTTACC AGCACTCGCA CCACCCGCCG GCGCCTGCTG 15100  
GGCGAGGAGG AGTACCTAAA CAACTCGCTG CTGCAGCCCG AGCGCGAAAA 15150  
AAACCTGCCT CCGGCATTTC CCAACAAACGG GATAGAGAGC CTAGTGGACA 15200  
AGATGAGTAG ATGGAAGACG TACGCGCAGG AGCACAGGGA CGTGCAGGCC 15250  
CCCGCGCCCGC CCACCCGTAG TCAAAGGCAC GACCGTCAGC GGGGCTGGT 15300  
GTGGGAGGAC GATGACTCGG CAGACGACAG CAGCGTCCTG GATTGGGAG 15350  
GGAGTGGCAA CCCGTTGCG CACCTTCGCC CCAGGCTGGG GAGAATGTTT 15400  
TAAAAAAA AAAGCATGAT GCAAAATAAA AAACTCACCA AGGCCATGGC 15450  
ACCGAGCGTT GGTTTCTTG TATTCCCCTT AGTATGCGGC GCGCGGCGAT 15500  
GTATGAGGAA GGTCCCTCTC CCTCCTACGA GAGTGTGGTG AGCGCGGCC 15550  
CAGTGGCGGC GGCGCTGGGT TCTCCCTCG ATGCTCCCT GGACCCGCCG 15600  
TTTGTGCCTC CGCGGTACCT GCGGCCTACC GGGGGGAGAA ACAGCATCCG 15650  
TTACTCTGAG TTGGCACCCC TATTGACACAC CACCCGTGTG TACCTGGTGG 15700  
ACAACAAAGTC AACGGATGTG GCATCCCTGA ACTACCAGAA CGACCCACAGC 15750  
AACTTCTGA CCACGGTCAT TCAAAACAAT GACTACAGCC CGGGGGAGGC 15800  
AAGCACACAG ACCATCAATC TTGACGACCG GTCGCACTGG GGCGCGACC 15850  
TGAAAACCAT CCTGCATACC AACATGCCAA ATGTGAACGA GTTCATGTTT 15900

ACCAATAAGT TTAAGGCGCG GGTGATGGTG TCGCGCTTGC CTACTAAGGA	15950
CAATCAGGTG GAGCTGAAAT ACGAGTGGGT GGAGTTCACG CTGCCCGAGG	16000
GCAACTACTC CGAGACCAGT ACCATAGACC TTATGAACAA CGCGATCGTG	16050
GAGCACTACT TGAAAGTGGG CAGACAGAAC GGGGTTCTGG AAAGCGACAT	16100
CGGGGTAAAG TTTGACACCC GCAACTTCAG ACTGGGGTTT GACCCCGTCA	16150
CTGGTCTTGT CATGCCCTGGG GTATATACAA ACGAAGCCTT CCATCCAGAC	16200
ATCATTTCGC TGCCAGGATG CGGGGTGGAC TTCACCCACA GCCGCCTGAG	16250
CAACTTGTG GGCATCCGCA AGCGGCAACC CTTCCAGGAG GGCTTTAGGA	16300
TCACCTACGA TGATCTGGAG GGTGGTAACA TTCCCGCACT GTTGGATGTG	16350
GACGCCTACC AGGCGAGCTT GAAAGATGAC ACCGAACAGG GCGGGGGTGG	16400
CGCAGGGCGGC AGCAACAGCA GTGGCAGCGG CGCGGAAGAG AACTCCAACG	16450
CGGCAGCCGC GGCAATGCAG CCGGTGGAGG ACATGAACGA TCATGCCATT	16500
CGCGGCGACA CCTTGCCAC ACGGGCTGAG GAGAAGCGCG CTGAGGCCGA	16550
AGCAGCGGCC GAAGCTGCCG CCCCCGCTGC GCAACCCGAG GTCGAGAACG	16600
CTCAGAAGAA ACCGGTGATC AAACCCCTGA CAGAGGACAG CAAGAAACGC	16650
AGTTACAACC TAATAAGCAA TGACAGCACC TTCACCCAGT ACCGCAGCTG	16700
GTACCTTGCA TACAACCTACG GCGACCCCTCA GACCGGAATC CGCTCATGGA	16750
CCCTGCTTTG CACTCCTGAC GTAACCTGCG GCTCGGAGCA GGTCTACTGG	16800
TCGTTGCCAG ACATGATGCA AGACCCCGTG ACCTTCCGCT CCACGCGCCA	16850
GATCAGCAAC TTTCCGGTGG TGGGCGCCGA GCTGTTGCCG GTGCACTCCA	16900
AGAGCTTCTA CAACGACCAG GCCGTCTACT CCCAACTCAT CCGCCAGTTT	16950
ACCTCTCTGA CCCACGTGTT CAATCGCTTT CCCGAGAAC AGATTTGGC	17000
GCGCCCGCCA GCCCCCACCA TCACCACCGT CAGTGAAAAC GTTCCCTGCTC	17050
TCACAGATCA CGGGACGCTA CCGCTGCGCA ACAGCATCGG AGGAGTCCAG	17100
CGAGTGACCA TTACTGACGC CAGACGCCGC ACCTGCCCCCT ACGTTTACAA	17150
GGCCCTGGGC ATAGTCTCGC CGCGCGTCCT ATCGAGCCGC ACTTTTGAG	17200

CAAGCATGTC	CATCCTTATA	TCGCCAGCA	ATAACACAGG	CTGGGGCCTG	17250
CGCTTCCCAA	GCAAGATGTT	TGGCGGGGCC	AAGAAGCGCT	CCGACCAACA	17300
CCCAGTGCAC	GTGCGCGGGC	ACTACCGCGC	GCCCTGGGC	GCGCACAAAC	17350
GCGGCCGAC	TGGGCGCACC	ACCGTCGATG	ACGCCATCGA	CGCGGTGGTG	17400
GAGGAGGCGC	GCAACTACAC	GCCCACGCCG	CCACCGATGT	CCACAGTGGA	17450
CGCGGCCATT	CAGACCGTGG	TGCGCGGAGC	CCGGCGCTAT	GCTAAAATGA	17500
AGAGACGGCG	GAGGCGCGTA	GCACGTCGCC	ACCGCCGCCG	ACCCGGCACT	17550
GCCGCCAAC	GCGCGCGGGC	GGCCCTGCTT	AACCGCGCAC	GTCGCACCGG	17600
CCGACGGCG	GCCATGCGGG	CCGCTCGAAG	GCTGGCCCG	GGTATTGTCA	17650
CTGTGCCCCC	CAGGTCCAGG	CGACGAGCGG	CCGCCGCAGC	AGCCGCGGCC	17700
ATTAGTGCTA	TGACTCAGGG	TCGCAGGGGC	AACGTGTATT	GGGTGCGCGA	17750
CTCGGTTAGC	GGCCTGCGCG	TGCCCGTGC	CACCCGCC	CCGCGCAACT	17800
AGATTGCAAG	AAAAAAACTAC	TTAGACTCGT	ACTGTTGTAT	GTATCCAGCG	17850
GCGCGGCCG	GCAACGAAGC	TATGTCCAAG	CGCAAAATCA	AAGAAGAGAT	17900
GCTCCAGGTC	ATCGCGCCGG	AGATCTATGG	CCCCCGAAG	AAGGAAGAGC	17950
AGGATTACAA	GCCCCGAAAG	CTAAAGCGGG	TCAAAAAGAA	AAAGAAAGAT	18000
GATGATGATG	AACTTGACGA	CGAGGTGGAA	CTGCTGCACG	CTACCGCGCC	18050
CAGGCGACGG	GTACAGTGGA	AAGGTGACG	CGTAAAACGT	TTTTGCGAC	18100
CCGGCACCAAC	CGTAGTCTTT	ACGCCCGGTG	AGCGCTCCAC	CCGCACCTAC	18150
AAGCGCGTGT	ATGATGAGGT	GTACGGCGAC	GAGGACCTGC	TTGAGCAGGC	18200
CAACGAGCGC	CTCGGGGAGT	TTGCCTACGG	AAAGCGGCAT	AAGGACATGC	18250
TGGCGTTGCC	GCTGGACGAG	GGCAACCAA	CACCTAGCCT	AAAGCCCGTA	18300
ACACTGCAGC	AGGTGCTGCC	CGCGCTTGCA	CCGTCCGAAG	AAAAGCGCGG	18350
CCTAAAGCGC	GAGTCTGGTG	ACTTGGCACC	CACCGTGCAG	CTGATGGTAC	18400
CCAAGCGCCA	GCGACTGGAA	GATGTCTTGG	AAAAAAATGAC	CGTGGAACCT	18450
GGGCTGGAGC	CCGAGGTCCG	CGTGCAGCCA	ATCAAGCAGG	TGGCGCCGGG	18500

ACTGGCGTG	CAGACCGTGG	ACGTTCAGAT	ACCCACTACC	AGTAGCACCA	18550
GTATTGCCAC	CGCCACAGAG	GGCATGGAGA	CACAAACGTC	CCCGGTTGCC	18600
TCAGCGGTGG	CGGATGCCGC	GGTGCAGGCG	GTCGCTGCCG	CCCGGTCCAA	18650
GACCTCTACG	GAGGTGCAAA	CGGACCCGTG	GATGTTTCGC	GTTCAGCCC	18700
CCCGGCGCCC	GCGCGGTTCG	AGGAAGTACG	GCGCCGCCAG	CGCGCTACTG	18750
CCCGAATATG	CCCTACATCC	TTCCATTGCG	CCTACCCCCG	GCTATCGTGG	18800
CTACACCTAC	CGCCCCAGAA	GACGAGCAAC	TACCCGACGC	CGAACCCACCA	18850
CTGGAACCCG	CCGCCGCCGT	CGCCGTGCC	AGCCCGTGCT	GGCCCCGATT	18900
TCCGTGCGCA	GGGTGGCTCG	CGAAGGAGGC	AGGACCCCTGG	TGCTGCCAAC	18950
AGCGCGCTAC	CACCCCAGCA	TCGTTAAAAA	GCCGGTCTTT	GTGGTTCTTG	19000
CAGATATGGC	CCTCACCTGC	CGCCTCCGTT	TCCCGGTGCC	GGGATTCCGA	19050
GGAAGAATGC	ACCGTAGGAG	GGGCATGGCC	GGCCACGGCC	TGACGGGC GG	19100
CATGCGTCGT	GCGCACCACC	GGCGGGGGCG	CGCGTCGCAC	CGTCGCATGC	19150
GCGCGGTAT	CCTGCCCTC	CTTATTCCAC	TGATGCCGC	GGCGATTGGC	19200
GCCGTGCCCG	GAATTGCATC	CGTGGCCTTG	CAGGCGCAGA	GACACTGATT	19250
AAAAACAAGT	TGCATGTGGA	AAAATCAAAA	AAAAAAAGTCT	GGACTCTCAC	19300
GCTCGCTTGG	TCCTGTAACT	ATTTTGTAGA	ATGGAAGACA	TCAACTTTGC	19350
GTCTCTGGCC	CCGCGACACG	GCTCGGCC	GTTCATGGGA	AACTGGCAAG	19400
ATATCGGCAC	CAGCAATATG	AGCGGTGGCG	CCTTCAGCTG	GGGCTCGCTG	19450
TGGAGCGGCA	TTAAAAATT	CGGTTCCACC	GTAAAGAACT	ATGGCAGCAA	19500
GGCCTGGAAC	AGCAGCACAG	GCCAGATGCT	GAGGGATAAG	TTGAAAGAGC	19550
AAAATTCCA	ACAAAAGGTG	GTAGATGGCC	TGGCCTCTGG	CATTAGCGGG	19600
GTGGTGGACC	TGGCCAACCA	GGCAGTGCAA	AATAAGATTA	ACAGTAAGCT	19650
TGATCCCCGC	CCTCCCGTAG	AGGAGCCTCC	ACCGGCCGTG	GAGACAGTGT	19700
CTCCAGAGGG	GCGTGGCGAA	AAGCGTCCGC	GCCCCGACAG	GGAAGAAACT	19750
CTGGTGACGC	AAATAGACGA	GCCTCCCTCG	TACGAGGAGG	CACTAAAGCA	19800

AGGCCTGCC	ACCACCCGTC	CCATCGCGCC	CATGGCTACC	GGAGTGCTGG	19850
GCCAGCACAC	ACCCGTAACG	CTGGACCTGC	CTCCCCCCGC	CGACACCCAG	19900
CAGAAACCTG	TGCTGCCAGG	CCCGACCGCC	GTTGTTGTA	CCCGTCCTAG	19950
CCGCGCGTCC	CTGCGCCGCG	CCGCCAGCGG	TCCGCGATCG	TTGCGGCCCG	20000
TAGCCAGTGG	CAACTGGCAA	AGCACACTGA	ACAGCATCGT	GGGTCTGGGG	20050
GTGCAATCCC	TGAAGCGCCG	ACGATGCTTC	TGAATAGCTA	ACGTGTCGTA	20100
TGTGTGTCAT	GTATGCGTCC	ATGTCGCCGC	CAGAGGAGCT	GCTGAGCCGC	20150
CGCGCGCCCG	CTTTCCAAGA	TGGCTACCCC	TTCGATGATG	CCGCAGTGGT	20200
CTTACATGCA	CATCTCGGGC	CAGGACGCCT	CGGAGTACCT	GAGCCCCGGG	20250
CTGGTGCAGT	TTGCCCGCGC	CACCGAGACG	TACTTCAGCC	TGAATAACAA	20300
GTTTAGAAC	CCCACGGTGG	CGCCTACGCA	CGACGTGACC	ACAGACCGGT	20350
CCCAGCGTTT	GACGCTGCAG	TTCATCCCTG	TGGACCGTGA	GGATACTGCG	20400
TACTCGTACA	AGGCGCGGTT	CACCCCTAGCT	GTGGGTGATA	ACCGTGTGCT	20450
GGACATGGCT	TCCACGTACT	TTGACATCCG	CGGCGTGCTG	GACAGGGGCC	20500
CTACTTTAA	GCCCTACTCT	GGCACTGCCT	ACAACGCCCT	GGCTCCCAAG	20550
GGTCCCCCAA	ATCCTTGCAG	ATGGGATGAA	GCTGCTACTG	CTCTTGAAAT	20600
AAACCTAGAA	GAAGAGGACG	ATGACAACGA	AGACGAAGTA	GACGAGCAAG	20650
CTGAGCAGCA	AAAAACTCAC	GTATTTGGGC	AGGCGCCTTA	TTCTGGTATA	20700
AATATTACAA	AGGAGGGTAT	TCAAATAGGT	GTCGAAGGTC	AAACACCTAA	20750
ATATGCCGAT	AAAACATTTC	AACCTGAACC	TCAAATAGGA	GAATCTCAGT	20800
GGTACGAAAC	TGAAATTAAT	CATGCAGCTG	GGAGAGTCCT	TAAAAAGACT	20850
ACCCCAATGA	AACCATGTTA	CGGTTCATAT	GCAAAACCCA	CAAATGAAAA	20900
TGGAGGGCAA	GGCATTCTTG	TAAAGCAACA	AAATGGAAAG	CTAGAAAGTC	20950
AAGTGGAAAT	GCAATTTC	TCAACTACTG	AGGCGACCGC	AGGCAATGGT	21000
GATAACTTGA	CTCCTAAAGT	GGTATTGTAC	AGTGAAGATG	TAGATATAGA	21050
AACCCCAGAC	ACTCATATTT	CTTACATGCC	CACTATTAAG	GAAGGTAACT	21100

CACGAGAACT	AATGGGCCAA	CAATCTATGC	CCAACAGGCC	TAATTACATT	21150
GCTTTAGGG	ACAATTTAT	TGGTCTAATG	TATTACAACA	GCACGGGTAA	21200
TATGGGTGTT	CTGGCGGCC	AAGCATCGCA	GTTGAATGCT	GTTGTAGATT	21250
TGCAAGACAG	AAACACAGAG	CTTTCATACC	AGCTTTGCT	TGATTCCATT	21300
GGTGATAGAA	CCAGGTACTT	TTCTATGTGG	AATCAGGCTG	TTGACAGCTA	21350
TGATCCAGAT	GTTAGAATTA	TTGAAAATCA	TGGAACGTGAA	GATGAACCTTC	21400
CAAATTACTG	CTTTCCACTG	GGAGGGTGTGA	TTAATACAGA	GACTCTTACC	21450
AAGGTAAAAC	CTAAAACAGG	TCAGGAAAAT	GGATGGGAAA	AAGATGCTAC	21500
AGAATTTCA	GATAAAAATG	AAATAAGAGT	TGGAATAAT	TTGCCATGG	21550
AAATCAATCT	AAATGCCAAC	CTGTGGAGAA	ATTCCTGTA	CTCCAACATA	21600
GCGCTGTATT	TGCCCGACAA	GCTAAAGTAC	AGTCCTTCCA	ACGTAAAAAT	21650
TTCTGATAAC	CCAAACACCT	ACGACTACAT	GAACAAGCGA	GTGGTGGCTC	21700
CCGGGTTAGT	GGACTGCTAC	ATTAACCTTG	GAGCACGCTG	GTCCCTTGAC	21750
TATATGGACA	ACGTCAACCC	ATTTAACACAC	CACCGCAATG	CTGGCCTGCG	21800
CTACCGCTCA	ATGTTGCTGG	GCAATGGTCG	CTATGTGCC	TTCCACATCC	21850
AGGTGCCTCA	GAAGTTCTTT	GCCATTAAAA	ACCTCCTTCT	CCTGCCGGGC	21900
TCATACACCT	ACGAGTGGAA	CTTCAGGAAG	GATGTTAAC	TGGTTCTGCA	21950
GAGCTCCCTA	GGAAATGACC	TAAGGGTTGA	CGGAGCCAGC	ATTAAGTTTG	22000
ATAGCATTG	CCTTACGCC	ACCTTCTTCC	CCATGGCCCA	CAACACCGCC	22050
TCCACGCTTG	AGGCCATGCT	TAGAAACGAC	ACCAACGACC	AGTCCTTAA	22100
CGACTATCTC	TCCGCCGCCA	ACATGCTCTA	CCCTATACCC	GCCAACGCTA	22150
CCAACGTGCC	CATATCCATC	CCCTCCCGCA	ACTGGGCGGC	TTTCCGCCGGC	22200
TGGGCCTTCA	CGGCCCTTAA	GACTAAGGAA	ACCCCATCAC	TGGGCTCGGG	22250
CTACGACCT	TATTACACCT	ACTCTGGCTC	TATACCCTAC	CTAGATGGAA	22300
CCTTTACCT	CAACCACACC	TTAAGAAGG	TGGCCATTAC	CTTGTACTCT	22350
TCTGTCAGCT	GGCCTGGCAA	TGACCGCCTG	CTTACCCCCA	ACGAGTTTGA	22400

AATTAAGCGC	TCAGTTGACG	GGGAGGGTTA	CAACGTTGCC	CAGTGTAACA	22450
TGACCAAAGA	CTGGTTCTG	GTACAAATGC	TAGCTAACTA	CAACATTGGC	22500
TACCAGGGCT	TCTATATCCC	AGAGAGCTAC	AAGGACCGCA	TGTACTCCTT	22550
CTTTAGAAAC	TTCCAGCCCA	TGAGCCGTCA	GGTGGTGGAT	GATACTAAAT	22600
ACAAGGACTA	CCAACAGGTG	GGCATCCTAC	ACCAACACAA	CAACTCTGGA	22650
TTTGTGGCT	ACCTTGCCCC	CACCATGCGC	GAAGGACAGG	CCTACCCCTGC	22700
TAACTTCCCC	TATCCGCTTA	TAGGCAAGAC	CGCAGTTGAC	AGCATTACCC	22750
AGAAAAAGTT	TCTTGCGAT	CGCACCCCTT	GGCGCATCCC	ATTCTCCAGT	22800
AACTTTATGT	CCATGGGCGC	ACTCACAGAC	CTGGGCCAAA	ACCTTCTCTA	22850
CGCCAACCTCC	GCCCACGCGC	TAGACATGAC	TTTGAGGTG	GATCCCATGG	22900
ACGAGCCCAC	CCTTCTTTAT	TTTTGTTTG	AAGTCTTG	CGTGGTCCGT	22950
GTGCACCGGC	CGCACCGCGG	CGTCATCGAA	ACCGTGTACC	TGCGCACGCC	23000
CTTCTCGGCC	GGCAACGCCA	CAACATAAAG	AAGCAAGCAA	CATCAACAAAC	23050
AGCTGCCGCC	ATGGGCTCCA	GTGAGCAGGA	ACTGAAAGCC	ATTGTCAAAG	23100
ATCTTGGTTG	TGGGCCATAT	TTTTGGGCA	CCTATGACAA	GCGCTTTCCA	23150
GGCTTTGTTT	CTCCACACAA	GCTCGCTGC	GCCATAGTCA	ATACGGCCGG	23200
TCGGGAGACT	GGGGCGTAC	ACTGGATGGC	CTTGCCTGG	AACCCGCACT	23250
CAAAAACATG	CTACCTCTT	GAGCCCTTG	GCTTTCTGA	CCAGCGACTC	23300
AAGCAGGTTT	ACCAGTTGA	GTACGAGTCA	CTCCTGCGCC	GTAGGCCAT	23350
TGCTTCTTCC	CCCGACCGCT	GTATAACGCT	GGAAAAGTCC	ACCCAAAGCG	23400
TACAGGGGCC	CAAECTGGCC	GCCTGTGGAC	TATTCTGCTG	CATGTTCTC	23450
CACGCCTTG	CCAACTGGCC	CCAAACTCCC	ATGGATCACA	ACCCCAACCAT	23500
GAACCTTATT	ACCGGGGTAC	CCAACTCCAT	GCTCAACAGT	CCCCAGGTAC	23550
AGCCCACCC	GCCTCGCAAC	CAGGAACAGC	TCTACAGCTT	CCTGGAGCGC	23600
CACTCGCCCT	ACTTCCGCAG	CCACAGTGC	CAGATTAGGA	GCGCCACTTC	23650
TTTTGTAC	TTGAAAAAC	TGTAAAATA	ATGTACTAGA	GACACTTTCA	23700

ATAAAGGCAA	ATGCTTTAT	TTGTACACTC	TCGGGTGATT	ATTTACCCCC	23750
ACCCTTGCCG	TCTGCGCCGT	TTAAAAATCA	AAGGGGTTCT	GCCGCGCATC	23800
GCTATGCGCC	ACTGGCAGGG	ACACGTTGCG	ATACTGGTGT	TTAGTGCTCC	23850
ACTTAAACCTC	AGGCACAACC	ATCCGGGCA	GCTCGGTGAA	GTTTCACTC	23900
CACAGGCTGC	GCACCATCAC	CAACCGTGT	AGCAGGTCGG	GCGCCGATAT	23950
CTTGAAGTCG	CAGTTGGGGC	CTCCGCCCTG	CGCGCGCGAG	TTGCGATACA	24000
CAGGGTTGCA	GCACGTGGAAC	ACTATCAGCG	CCGGGTGGTG	CACGCTGGCC	24050
AGCACGCTCT	TGTCGGAGAT	CAGATCCGCG	TCCAGGTCT	CCGCGTTGCT	24100
CAGGGCGAAC	GGAGTCAACT	TTGGTAGCTG	CCTTCCCAAA	AAGGGCGCGT	24150
GCCCAGGCTT	TGAGTTGCAC	TCGCACCGTA	GTGGCATCAA	AAGGTGACCG	24200
TGCCCAGGTCT	GGCGTTAGG	ATACAGCGCC	TGCATAAAAG	CCTTGATCTG	24250
CTTAAAAGCC	ACCTGAGCCT	TTGCGCCTTC	AGAGAAGAAC	ATGCCGCAAG	24300
ACTTGCCGGA	AAACTGATTG	GCCGGACAGG	CCGCGTCGTG	CACGCAGCAC	24350
CTTGCAGTCGG	TGTTGGAGAT	CTGCACCAACA	TTTCGGCCCC	ACCGGTTCTT	24400
CACGATCTTG	GCCTTGCTAG	ACTGCTCCTT	CAGCGCGCGC	TGCCCGTTTT	24450
CGCTCGTCAC	ATCCATTCA	ATCACGTGCT	CCTTATTTAT	CATAATGCTT	24500
CCGTGTAGAC	ACTTAAGCTC	GCCTTCGATC	TCAGCGCAGC	GGTGCAGCCA	24550
CAACGCGCAG	CCCGTGGGCT	CGTGATGCTT	GTAGGTCACC	TCTGCAAACG	24600
ACTGCAGGTA	CGCCTGCAGG	AATCGCCCCA	TCATCGTCAC	AAAGGTCTTG	24650
TTGCTGGTGA	AGGTCAAGCTG	CAACCCGCGG	TGCTCCTCGT	TCAGCCAGGT	24700
CTTGCATACG	GCCGCCAGAG	CTTCCACTTG	GTCAGGCAGT	AGTTTGAAGT	24750
TCGCCCTTAG	ATCGTTATCC	ACGTGGTACT	TGTCCATCAG	CGCGCGCGCA	24800
GCCTCCATGC	CCTTCTCCCA	CGCAGACACG	ATCGGCACAC	TCAGCGGGTT	24850
CATCACCGTA	ATTTCACTTT	CCGCTTCGCT	GGGCTCTTCC	TCTTCCTCTT	24900
GCGTCCGCAT	ACCACGCGCC	ACTGGGTCGT	CTTCATTCAAG	CCGCCGCACT	24950
GTGCGCTTAC	CTCCTTGCC	ATGCTTGATT	AGCACCGGTG	GGTTGCTGAA	25000

ACCCACCATT	TGTAGCGCCA	CATCTTCTCT	TTCTTCCTCG	CTGTCCACGA	25050
TTACCTCTGG	TGATGGCGGG	CGCTCGGGCT	TGGGAGAAAGG	GCGCTTCTTT	25100
TTCTTCTTGG	GCGCAATGGC	CAAATCCGCC	GCCGAGGTCTG	ATGGCCGCAGG	25150
GCTGGGTGTG	CGCGGCACCA	GCGCGTCTTG	TGATGAGTCT	TCCTCGTCCT	25200
CGGACTCGAT	ACGCCGCCTC	ATCCGCTTTT	TTGGGGCGCG	CCGGGGAGGC	25250
GGCGCGACGG	GGGACGGGGA	CGACACGTCC	TCCATGGTTG	GGGGACGTCTG	25300
CGCCGCACCG	CGTCCCGCCT	CGGGGGTGGT	TTCGCGCTGC	TCCTCTTCCC	25350
GAATGGCCAT	TTCCCTCTCC	TATAGGCAGA	AAAAGATCAT	GGAGTCAGTC	25400
GAGAAGAAGG	ACAGCCTAAC	CGCCCCCTCT	GAGTCGCCA	CCACCGCCTC	25450
CACCGATGCC	GCCAACGCGC	CTACCACCTT	CCCCGTCTGAG	GCACCCCCGC	25500
TTGAGGAGGA	GGAAGTGATT	ATCGAGCAGG	ACCCAGGTTT	TGTAAGCGAA	25550
GACGACGAGG	ACCGCTCAGT	ACCAACAGAG	GATAAAAAGC	AAGACCAGGA	25600
CAACCGAGAG	GCAAACGAGG	AACAAGTCGG	GCGGGGGGAC	GAAAGGCATG	25650
GCGACTACCT	AGATGTGGGA	GACGACGTGC	TGTTGAAGCA	TCTGCAGCGC	25700
CAGTGCCTCA	TTATCTGCCTA	CGCGTTGCAA	GAGCGCAGCG	ATGTGCCCT	25750
CGCCATAGCG	GATGTCAGCC	TTGCCTACGA	ACGCCACCTA	TTCTCACCGC	25800
GCGTACCCCC	CAAACGCCAA	GAAAACGGCA	CATGCGAGCC	CAACCCGCAGC	25850
CTCAACTTCT	ACCCCGTATT	TGCCGTGCCA	GAGGTGCTTG	CCACCTATCA	25900
CATCTTTTTC	CAAAACTGCA	AGATACCCCT	ATCCTGCCGT	GCCAACCGCA	25950
GCCGAGCGGA	CAAGCAGCTG	GCCTTGCAGGC	AGGGCGCTGT	CATACTGAT	26000
ATCGCCTCGC	TCAACGAAGT	GCCAAAATC	TTTGAGGGTC	TTGGACGCGA	26050
CGAGAAGCGC	GCGGCAAACG	CTCTGCAACA	GGAAAACAGC	GAAAATGAAA	26100
GTCACTCTGG	AGTGTGAGGTG	GAACTCGAGG	GTGACAAACGC	GCGCCTAGCC	26150
GTACTAAAC	GCAGGCATCGA	GGTCACCCAC	TTTGCCTACC	CGGCACCTAA	26200
CCTACCCCCC	AAGGTCTGAA	GCACAGTCAT	GAGTGAGCTG	ATCGTGCCTC	26250
GTGCGCAGCC	CCTGGAGAGG	GATGCAAATT	TGCAAGAACAA	AACAGAGGAG	26300

GGCCTACCCG	CAGTGGCGA	CGAGCAGCTA	GCGCGCTGGC	TTCAAAACGCG	26350
CGAGCCTGCC	GACTGGAGG	AGCGACGCAA	ACTAATGATG	GCCGCAGTGC	26400
TCGTTACCGT	GGAGCTTGAG	TGCATGCAGC	GGTTCTTGC	TGACCCGGAG	26450
ATGCAGCGCA	AGCTAGAGGA	AACATTGCAC	TACACCTTC	GACAGGGCTA	26500
CGTACGCCAG	GCCTGCAAGA	TCTCCAACGT	GGAGCTCTGC	AACCTGGTCT	26550
CCTACCTTGG	AATTTGCAC	GAAAACGCC	TTGGGCAAAA	CGTGCTTCAT	26600
TCCACGCTCA	AGGGCGAGGC	GCGCCGCGAC	TACGTCCGCG	ACTGCCTTA	26650
CTTATTCTA	TGCTACACCT	GGCAGACGGC	CATGGCGTT	TGGCAGCAGT	26700
GCTTGGAGGA	GTGCAACCTC	AAGGAGCTGC	AGAAAATGCT	AAAGCAAAAC	26750
TTGAAGGACC	TATGGACGGC	CTTCAACGAG	CGCTCCGTGG	CCGCGCACCT	26800
GGCGGACATC	ATTTCCCCG	AACGCCTGCT	TAAAACCCCTG	CAACAGGGTC	26850
TGCCAGACTT	CACCAAGTCAA	AGCATGTTGC	AGAACTTTAG	GAACCTTATC	26900
CTAGAGCGCT	CAGGAATCTT	GCCCCCACC	TGCTGTGCAC	TTCCTAGCGA	26950
CTTGTGCC	ATTAAGTACC	GCGAATGCC	TCCGCCGCTT	TGGGGCCACT	27000
GCTACCTTCT	GCAGCTAGCC	AACTACCTTG	CCTACCACTC	TGACATAATG	27050
GAAGACGTGA	GCGGTGACGG	TCTACTGGAG	TGTCACTGTC	GCTGCAACCT	27100
ATGCACCCCG	CACCGCTCCC	TGGTTGCAA	TTCGCAGCTG	CTTAACGAAA	27150
GTCAAATTAT	CGGTACCTTT	GAGCTGCAGG	GTCCCTCGCC	TGACGAAAAG	27200
TCCGGGCTC	CGGGGTTGAA	ACTCACTCCG	GGGCTGTGGA	CGTCGGCTTA	27250
CCTTCGCAA	TTTGTACCTG	AGGACTACCA	CGCCCACGAG	ATTAGGTTCT	27300
ACGAAGACCA	ATCCCCCCG	CCAAATGCGG	AGCTTACCGC	CTGCGTCATT	27350
ACCCAGGGCC	ACATTCTTGG	CCAATTGCAA	GCCATCAACA	AAGCCCGCCA	27400
AGAGTTCTG	CTACGAAAGG	GACGGGGGGT	TTACTTGGAC	CCCCAGTCCG	27450
GCGAGGAGCT	CAACCCAATC	CCCCCGCCGC	CGCAGCCCTA	TCAGCAGCAG	27500
CCGCAGGGCCC	TTGCTTCCCA	GGATGGCACC	AAAAAAGAAG	CTGCAGCTGC	27550
CGCCGCCACC	CACGGACGAG	GAGGAATACT	GGGACAGTCA	GGCAGAGGAG	27600

GT	TTTGGACG	AGGAGGAGGA	GGACATGATG	GAAGACTGGG	AGAGCCTAGA	27650
CG	AGGAAGCT	TCCGAGGTCTG	AAGAGGTGTC	AGACGAAACA	CCGTCACCCT	27700
CG	GTGCGATT	CCCCTCGCCG	GCGCCCCAGA	AATCGGCAAC	CGGTTCCAGC	27750
AT	GGCTACAA	CCTCCGCTCC	TCAGGGCGCCG	CCGGCACTGC	CCGTTCGCCG	27800
AC	CCCAACCGT	AGATGGGACA	CCACTGGAAC	CAGGGCCGGT	AAGTCCAAGC	27850
AG	CCGCCGCC	GTAGCCCAA	GAGCAACAAAC	AGCGCCAAGG	CTACCGCTCA	27900
TG	GGCGCGGGC	ACAAGAACGC	CATAGTTGCT	TGCTTGCAAG	ACTGTGGGGG	27950
CA	ACATCTCC	TTCGCCCGCC	GCTTTCTTCT	CTACCATCAC	GGCGTGGCCT	28000
TC	CCCCCGTAA	CATCCTGCAT	TACTACCGTC	ATCTCTACAG	CCCATACTGC	28050
AC	CCGGCGGCA	GCGGCAGCGG	CAGCAACAGC	AGCGGCCACA	CAGAAGCAAA	28100
GG	CGACCGGA	TAGCAAGACT	CTGACAAAGC	CCAAGAAATC	CACAGCGGCG	28150
GC	AGCAGCAG	GAGGAGGAGC	GCTGCGTCTG	GCGCCCAACG	AACCCGTATC	28200
GA	CCCCGGAG	CTTAGAAACA	GGATTTTCC	CACTCTGTAT	GCTATATTTC	28250
AA	CACAGAGCAG	GGGCCAAGAA	CAAGAGCTGA	AAATAAAAAA	CAGGTCTCTG	28300
CG	ATCCCTCA	CCCGCAGCTG	CCTGTATCAC	AAAAGCGAAG	ATCAGCTTCG	28350
GC	GCACGCTG	GAAGACGCGG	AGGCTCTCTT	CAGTAAATAC	TGCGCGCTGA	28400
CT	CTTAAGGA	CTAGTTTCGC	GCCCTTCTC	AAATTTAACG	GCGAAAACTA	28450
CG	TGTCATCTCC	AGCGGCCACA	CCC GGCGCCA	GCACCTGTCTG	TCAGCGCCAT	28500
TA	TATGAGCAAG	GAAATTCCCA	CGCCCTACAT	GTGGAGTTAC	CAGCCACAAA	28550
TG	GGGACTTGC	GGCTGGAGCT	GCCCAAGACT	ACTCAACCCG	AATAAACTAC	28600
AT	GAGCGCGG	GACCCCACAT	GATATCCCGG	GTCAACGGAA	TCCGCGCCCA	28650
CC	CGAAACCGA	ATTCTCTTGG	AACAGGGCGC	TATTACCACC	ACACCTCGTA	28700
AT	AAACCTTAA	TCCCCGTAGT	TGGCCCGCTG	CCCTGGTGT	CCAGGAAAGT	28750
CC	CGCTCCCA	CCACTGTGGT	ACTTCCCAGA	GACGCCAGG	CCGAAGTTCA	28800
GA	TGACTAAC	TCAGGGCGC	AGCTTGCAGG	CGGCTTCGT	CACAGGGTGC	28850
GG	TGCCCCGG	GCAGGGTATA	ACTCACCTGA	CAATCAGAGG	GCGAGGTATT	28900

CAGCTCAACG	ACGAGTCGGT	GAGCTCCTCG	CTTGGTCTCC	GTCCGGACGG	28950
GACATTTCAAG	ATCGGCAGCG	CCGGCCGTCC	TTCATTACAG	CCTCGTCAGG	29000
CAATCCTAAC	TCTGCAGACC	TCGTCCCTTG	AGCCGCGCTC	TGGAGGCATT	29050
GGAACCTCTGC	AATTATTGAA	GGAGTTGTG	CCATCGGTCT	ACTTTAACCC	29100
CTTCTCGGGA	CCTCCCGGCC	ACTATCCGA	TCAATTATT	CCTAACCTTG	29150
ACGCGGTAAA	GGACTCGGCG	GACGGCTACG	ACTGAATGTT	AAGTGGAGAG	29200
GCAGAGCAAC	TGCGCCTGAA	ACACCTGGTC	CACTGTCGCC	GCCACAAAGTG	29250
CTTTGCCCGC	GAETCCGGTG	AGTTTGCTA	CTTTGAATTG	CCCGAGGATC	29300
ATATCGAGGG	CCCAGCGCAC	GGCGTCCGGC	TTACCGCCCA	GGGAGAGCTT	29350
GCCCCGTAGCC	TGATTGGGA	GTTTACCCAG	CGCCCCCTGC	TAGTTGAGCG	29400
GGACAGGGGA	CCCTGTGTT	TCACTGTGAT	TTGCAACTGT	CCTAACCTTG	29450
GATTACATCA	AGATCTTGT	TGCCATCTCT	GTGCTGAGTA	TAATAAATAC	29500
AGAAAATAAA	ATATACTGGG	GCTCCTATCG	CCATCCTGTA	AACGCCACCG	29550
TCTTCACCCG	CCCAAGCAAA	CCAAGGCAGA	CCTTACCTGG	TACTTTAAC	29600
ATCTCTCCCT	CTGTGATT	CAACAGTTTC	AAACCCAGACG	GAGTGAGTCT	29650
ACGAGAGAAC	CTCTCCGAGC	TCAGCTACTC	CATCAGAAAA	AACACCACCC	29700
TCCTTACCTG	CCGGGAACGT	ACGAGTGCAGT	CACCGGCCGC	TGCACCAACAC	29750
CTACCGCCTG	ACCGTAAACC	AGACTTTTC	CGGACAGACC	TCAATAACTC	29800
TGTTTACCAAG	AACAGGAGGT	GAGCTTAGAA	AACCCTTAGG	GTATTAGGCC	29850
AAAGGCGCAG	CTACTGTGGG	GTTTATGAAC	AATTCAAGCA	ACTCTACGGG	29900
CTATTCTAAT	TCAGGTTCT	CTAGAATCGG	GGTTGGGGTT	ATTCTCTGTC	29950
TTGTGATTCT	CTTTATTCTT	ATACTAACGC	TTCTCTGCCT	AAGGCTCGCC	30000
GCCTGCTGTG	TGCACATTG	CATTTATTGT	CAGCTTTTA	AACGCTGGGG	30050
TCGCCACCCA	AGATGATTAG	GTACATAATC	CTAGGTTAC	TCACCCCTGTC	30100
GTCAGCCCAC	GGTACCCACCC	AAAAGGTGGA	TTTAAGGAG	CCAGCCTGTA	30150
ATGTTACATT	CGCAGCTGAA	GCTAATGAGT	GCACCACTCT	TATAAAATGC	30200

ACACAGAAC ATGAAAAGCT GCTTATTGCG CACAAAACA AAATTGGCAA	30250
GTATGCTGTT TATGCTATTT GGCAGCCAGG TGACACTACA GAGTATAATG	30300
TTACAGTTTT CCAGGGTAAA AGTCATAAAA CTTTTATGTA TACTTTCCA	30350
TTTTATGAAA TGTGCGACAT TACCATGTAC ATGAGCAAAC AGTATAAGTT	30400
GTGGCCCCCA CAAAATTGTG TGGAAAACAC TGGCACTTC TGCTGCACTG	30450
CTATGCTAAT TACAGTGCTC GCTTTGGTCT GTACCCCTACT CTATATTAAA	30500
TACAAAAGCA GACGCAGCTT TATTGAGGAA AAGAAAATGC CTTAATTAC	30550
TAAGTTACAA AGCTAATGTC ACCACTAACT GCTTTACTCG CTGCTTGCAA	30600
AACAAATTCA AAAAGTTAGC ATTATAATTA GAATAGGATT TAAACCCCCC	30650
GGTCATTCC TGCTCAATAC CATTCCCTG AACAAATTGAC TCTATGTGGG	30700
ATATGCTCCA GCGCTACAAC CTTGAAGTCA GGCTTCCTGG ATGTCAGCAT	30750
CTGACTTTGG CCAGCACCTG TCCCGCGGAT TTGTTCCAGT CCAACTACAG	30800
CGACCCACCC TAACAGAGAT GACCAACACA ACCAACGCGG CGCCGCTAC	30850
CGGACTTACA TCTACCACAA ATACACCCCA AGTTTCTGCC TTTGTCAATA	30900
ACTGGGATAA CTTGGGCATG TGGTGGTTCT CCATAGCGCT TATGTTGTA	30950
TGCCTTATTA TTATGTGGCT CATCTGCTGC CTAAAGCGCA AACCGCGCCG	31000
ACCACCCATC TATAGTCCCA TCATTGTGCT ACACCCAAAC AATGATGGAA	31050
TCCATAGATT GGACGGACTG AAACACATGT TCTTTCTCT TACAGTATGA	31100
TTAAATGAGA CATGATTCCCT CGAGTTTTA TATTACTGAC CCTTGTGCG	31150
CTTTTTGTG CGTGCTCCAC ATTGGCTGCG GTTTCTACA TCGAAGTAGA	31200
CTGCATTCCA GCCTCACAG TCTATTGCT TTACGGATT GTCACCCCTCA	31250
CGCTCATCTG CAGCCTCATC ACTGTGGTCA TCGCCTTAT CCAGTGCATT	31300
GACTGGGTCT GTGTGCGCTT TGCATATCTC AGACACCAC CCCAGTACAG	31350
GGACAGGACT ATAGCTGAGC TTCTTAGAAT TCTTTAATTA TGAAATTTAC	31400
TGTGACTTTT CTGCTGATTA TTTGCACCCCT ATCTGCGTT TGTTCCCCGA	31450
CCTCCAAGCC TCAAAGACAT ATATCATGCA GATTCACTCG TATATGGAAT	31500

ATTCCAAGTT GCTACAATGA AAAAAGCGAT CTTTCCGAAG CCTGGTTATA 31550  
TCCAATCATC TCTGTTATGG TGTTCTGCAG TACCATCTTA GCCCTAGCTA 31600  
TATATCCCTA CCTTGACATT GGCTGGAAAC GAATAGATGC CATGAACCAC 31650  
CCAACTTCC CGCGGCCCGC TATGCTTCCA CTGCAACAAG TTGTTGCCGG 31700  
CGGCTTTGTC CCAGCCAATC AGCCTCGCCC CACTTCTCCC ACCCCCAC 31750  
AAATCAGCTA CTTAATCTA ACAGGAGGAG ATGACTGACA CCCTAGATCT 31800  
AGAAATGGAC GGAATTATTA CAGAGCAGCG CCTGCTAGAA AGACGCAGGG 31850  
CAGCGGCCGA GCAACAGCGC ATGAATCAAG AGCTCCAAGA CATGGTTAAC 31900  
TTGCACCAGT GCAAAAGGGG TATCTTTGT CTGGTAAAGC AGGCCAAAGT 31950  
CACCTACGAC AGTAATACCA CGGGACACCG CCTTAGCTAC AAGTTGCCAA 32000  
CCAAGCGTCA GAAATTGGTG GTCATGGTGG GAGAAAAGCC CATTACCATA 32050  
ACTCAGCACT CGGTAGAAAC CGAAGGCTGC ATTCACTCAC CTTGTCAAGG 32100  
ACCTGAGGAT CTCTGCACCC TTATTAAGAC CCTGTGCGGT CTCAAAGATC 32150  
TTATTCCCTT TAACTAATAA AAAAAAATAA TAAAGCATCA CTTACTTAAA 32200  
ATCAGTTAGC AAATTCTGT CCAGTTTATT CAGCAGCACC TCCTTGCCCT 32250  
CCTCCCAGCT CTGGTATTGC AGCTTCCCTCC TGGCTGCAAA CTTTCTCCAC 32300  
AATCTAAATG GAATGTCAGT TTCCTCCTGT TCCTGTCCAT CCGCACCCAC 32350  
TATCTTCATG TTGTTGCAGA TGAAGCGCGC AAGACCGTCT GAAGATAACCT 32400  
TCAACCCCGT GTATCCATAT GACACGGAAA CCGGTCCCTCC AACTGTGCCT 32450  
TTTCTTACTC CTCCCTTTGT ATCCCCAAT GGGTTCAAG AGAGTCCCCC 32500  
TGGGGTACTC TCTTGCGCC TATCCGAACC TCTAGTTACC TCCAATGGCA 32550  
TGCTTGCCTC CAAAATGGGC AACGGCCTCT CTCTGGACGA GGCGGCAAC 32600  
CTTACCTCCC AAAATGTAAC CACTGTGAGC CCACCTCTCA AAAAAACCAA 32650  
GTCAAACATA AACCTGGAAA TATCTGCACC CCTCACAGTT ACCTCAGAAG 32700  
CCCTAACTGT GGCTGCCGCC GCACCTCTAA TGGTCGCGGG CAACACACTC 32750  
ACCATGCAAT CACAGGCCCG GCTAACCGTG CACGACTCCA AACTTAGCAT 32800

TGCCACCCAA GGACCCCTCA CAGTGTAGA AGGAAAGCTA GCCCTGCAAA 32850  
CATCAGGCC CCTCACCAAC ACCGATAGCA GTACCCTTAC TATCACTGCC 32900  
TCACCCCTC TAACTACTGC CACTGGTAGC TTGGGCATTG ACTTGAAAGA 32950  
GCCCATTTAT ACACAAAATG GAAAACCTAGG ACTAAAGTAC GGGGCTCCTT 33000  
TGCATGTAAC AGACGACCTA AACACTTGA CCGTAGCAAC TGGTCCAGGT 33050  
GTGACTATTA ATAATACTTC CTTGCAAAC ACTAAGTTACTG GAGCCTTGGG 33100  
TTTGATTCA CAAGGCAATA TGCAACTTAA TGTAGCAGGA GGACTAAGGA 33150  
TTGATTCTCA AAACAGACGC CTTATACTTG ATGTTAGTTA TCCGTTTGAT 33200  
GCTCAAAACC AACTAAATCT AAGACTAGGA CAGGGCCCTC TTTTTATAAA 33250  
CTCAGCCCAC AACTTGGATA TTAACTACAA CAAAGGCCTT TACCTGTTA 33300  
CAGCTTCAAA CAATTCCAAA AAGCTTGAGG TTAACCTAAG CACTGCCAAG 33350  
GGGTTGATGT TTGACGCTAC AGCCATAGCC ATTAATGCAG GAGATGGGCT 33400  
TGAATTTGGT TCACCTTAATG CACCAAAACAC AAATCCCCTC AAAACAAAAA 33450  
TTGGCCATGG CCTAGAATTG GATTCAAACA AGGCTATGGT TCCTAAACTA 33500  
GGAACCTGGCC TTAGTTTGAG CAGCACAGGT GCCATTACAG TAGGAAACAA 33550  
AAATAATGAT AAGCTAACTT TGTGGACCAAC ACCAGCTCCA TCTCCTAACT 33600  
GTAGACTAAA TGCAGAGAAA GATGCTAAC TCACTTTGGT CTTAACAAAA 33650  
TGTGGCAGTC AAATACTTGC TACAGTTCA GTTTGGCTG TTAAAGGCAG 33700  
TTTGGCTCCA ATATCTGGAA CAGTTCAAAG TGCTCATCTT ATTATAAGAT 33750  
TTGACGAAAA TGGAGTGCTA CTAAACAATT CCTTCCTGGA CCCAGAATAT 33800  
TGGAACTTTA GAAATGGAGA TCTTACTGAA GGCACAGCCT ATACAAACGC 33850  
TGTGGATTT ATGCCTAACCT TATCAGCTTA TCCAAAATCT CACGGTAAAA 33900  
CTGCCAAAAG TAACATTGTC AGTCAAGTTT ACTTAAACGG AGACAAAAC 33950  
AAACCTGTAA CACTAACCAT TACACTAAAC GGTACACAGG AAACAGGAGA 34000  
CACAACCTCCA AGTGCATACT CTATGTCATT TTCATGGGAC TGGTCTGGCC 34050  
ACAAACTACAT TAATGAAATA TTTGCCACAT CCTCTTACAC TTTTCATAC 34100

100

ATTGCCAAG AATAAAGAAT CGTTGTGTT ATGTTCAAC GTGTTATTT	34150
TTCAATTGCA GAAAATTCA AGTCATTTT CATTCACTAG TATAGCCCCA	34200
CCACCACTATA GCTTATACAG ATCACCGTAC CTTAATCAA CTCACAGAAC	34250
CCTAGTATTAC AACCTGCCAC CTCCCTCCA ACACACAGAG TACACAGTCC	34300
TTTCTCCCCG GCTGGCCTTA AAAAGCATCA TATCATGGGT AACAGACATA	34350
TTCTTAGGTG TTATATTCCA CACGGTTCC TGTCGAGCCA AACGCTCATC	34400
AGTGATATTA ATAAACTGGC GGCGATATAA AATGCAAGGT GCTGCTCAA	34450
AAATCAGGCA AAGCCTCGCG CAAAAAAAGAA AGCACATCGT AGTCATGCTC	34500
ATGCAGATAA AGGCAGGTAA GCTCCGGAAC CACCACAGAA AAAGACACCA	34550
TTTTCTCTC AAACATGTCT GCAGGTTTCT GCATAAACAC AAAATAAAAT	34600
AACAAAAAAA CATTAAACA TTAGAAGCCT GTCTTACAAC AGGAAAAACA	34650
ACCTTATAA GCATAAGACG GACTACGGCC ATGCCGGCGT GACCGTAAA	34700
AAACTGGTCA CCGTGATTAA AAAGCACCAC CGACAGCTCC TCGGTATGT	34750
CCGGAGTCAT AATGTAAGAC TCGGTAAACA CATCAGGTTG ATTCACTCGGT	34800
CAGTGCTAAA AAGCGACCGA AATAGCCCGG GGGAAATACAT ACCCGCAGGC	34850
GTAGAGACAA CATTACAGCC CCCATAGGAG GTATAACAAA ATTAATAGGA	34900
GAGAAAAACA CATAAACACC TGAAAAACCC TCCTGCCTAG GCAAAATAGC	34950
ACCCCTCCGC TCCAGAACAA CATAACAGCGC TTCACAGCGG CAGCCTAAC	35000
GTCAGCCTTA CCAGTAAAAA AGAAAACCTA TTAAAAAAAC ACCACTCGAC	35050
ACGGCACCAG CTCAATCAGT CACAGTGTAA AAAAGGGCCA AGTGCAGAGC	35100
GAGTATATAT AGGACTAAAA AATGACGTAA CGGTTAAAGT CCACAAAAAA	35150
CACCCAGAAA ACCGCACGCG AACCTACGCC CAGAAACGAA AGCCAAAAAA	35200
CCCACAACTT CCTCAAATCG TCACCTCCGT TTTCCCACGT TACGTAAC	35250
CCCATTAA GAAACTACA ATTCCCAACA CATAACAAGTT ACTCCGCCCT	35300
AAAACCTACG TCACCCGCC CGTTCCACG CCCCCGCGCCA CGTCACAAAC	35350

101

TCCACCCCT	CATTATCATA	TTGGCTTC	AA	TCCAAAATAA	GGTATATTAT	35400
TGATGATG						35408

## (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8509 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCCAATACG	CAAACCGCCT	CTCCCCGCGC	GTTGGCCGAT	TCATTAATGC	50
AGCTGCGCGC	TCGCTCGCTC	ACTGAGGCCG	CCCAGGGAAA	GCCCGGGCGT	100
CGGGCGACCT	TTGGTCGCCC	GGCCTCAGTG	AGCGAGCGAG	CGCCGAGAGA	150
GGGAGTGGCC	AACTCCATCA	CTAGGGGTT	CTTGTAGTTA	ATGATTAACC	200
CGCCATGCTA	CTTATCTACG	TAGCCATTCT	CTAGCCCCTG	CAGGTCGTTA	250
CATAACTTAC	GGTAAATGGC	CCGCCTGGCT	GACCGCCCAA	CGACCCCCGC	300
CCATTGACGT	CAATAATGAC	GTATGTTCCC	ATAGTAACGC	CAATAGGGAC	350
TTTCCATTGA	CGTCAATGGG	TGGAGTATTT	ACGGTAAACT	GCCCACITGG	400
CAGTACATCA	AGTGTATCAT	ATGCCAAGTA	CGCCCCCTAT	TGACGTCAAT	450
GACGGTAAAT	GGCCCGCCTG	GCATTATGCC	CAGTACATGA	CCTTATGGGA	500
CTTCCTACT	TGGCAGTACA	TCTACGTATT	AGTCATCGCT	ATTACCATGG	550
TGATGCGGTT	TTGGCAGTAC	ATCAATGGC	GTGGATAGCG	GTTTGACTCA	600
CGGGGATTTC	CAAGTCTCCA	CCCCATTGAC	GTCAATGGGA	GTTTGTITTC	650
GCACCAAAAT	CAACGGGACT	TTCCAAAATG	TCGTAACAAC	TCCGCCCCAT	700
TGACGCAAAT	GGCCGGTAGG	CGTGTACGGT	GGGAGGTCTA	TATAAGCAGA	750
GCTCGTTTAG	TGAACCGTCA	GATGCCCTGG	AGACGCCATC	CACGCTGTTT	800
TGACCTCCAT	AGAAGACACC	GGGACCGATC	CAGCCTCCGG	ACTCTAGAGG	850
ATCCGGTACT	CGAGGAACTG	AAAAACCAGA	AAGTTAACTG	GTAAGTTAG	900

102

TCTTTTGTC	TTTATTCA	GGTCCCGGAT	CCGGTGGTGG	TGCAAATCAA	950
AGAACTGCTC	CTCAGTGGAT	GTTGCCTTAA	CTTCTAGGCC	TGTACGGAAG	1000
TGTTACTTCT	GCTCTAAAAG	CTGCGGAATT	GTACCCGCGG	CCGCAATTCC	1050
CGGGGATCGA	AAGAGCCTGC	TAAAGCAAAA	AAGAAGTCAC	CATGTCGTTT	1100
ACTTTGACCA	ACAAGAACGT	GATTTCGTT	GCCGGTCTGG	GAGGCATTGG	1150
TCTGGACACC	AGCAAGGAGC	TGCTCAAGCG	CGATCCCGTC	GTTTACAAC	1200
GTCGTGACTG	GGAAAACCT	GGCGTTACCC	AACTTAATCG	CCTTGCAGCA	1250
CATCCCCCTT	TCGCCAGCTG	GCGTAATAGC	GAAGAGGCC	GCACCGATCG	1300
CCCTTCCCAA	CAGTTGCGCA	GCCTGAATGG	CGAATGGCGC	TTTGCCTGGT	1350
TTCCGGCACC	AGAACGGGTG	CCGGAAAGCT	GGCTGGAGTG	CGATCTTCCT	1400
GAGGCCGATA	CTGTCGTCGT	CCCCTCAAAC	TGGCAGATGC	ACGGTTACGA	1450
TGCGCCCATC	TACACCAACG	TAACCTATCC	CATTACGGTC	AATCCGCCGT	1500
TTGTTCCCAC	GGAGAAATCCG	ACGGGTTGTT	ACTCGCTCAC	ATTTAATGTT	1550
GATGAAAGCT	GGCTACAGGA	AGGCCAGACG	CGAATTATTT	TTGATGGCGT	1600
TAACTCGGCG	TTTCATCTGT	GGTGCAACGG	GCGCTGGTC	GGTTACGGCC	1650
AGGACAGTCG	TTGCCGTCT	GAATTTGACC	TGAGCGCATT	TTTACGCGCC	1700
GGAGAAAACC	GCCTCGCGGT	GATGGTCTG	CGTTGGAGTG	ACGGCAGTTA	1750
TCTGGAAGAT	CAGGATATGT	GGCGGATGAG	CGGCATTTTC	CGTGACGTCT	1800
CGTTGCTGCA	TAAACCGACT	ACACAAATCA	GCGATTTCCA	TGTTGCCACT	1850
CGCTTTAATG	ATGATTCAG	CCCGCTGTA	CTGGAGGCTG	AAGTTCAGAT	1900
GTGCGGCGAG	TTGCGTGA	ACTACGGGT	AACAGTTCT	TTATGGCAGG	1950
GTGAAACGCA	GGTCGCCAGC	GGCACCGCGC	CTTCGGCGG	TGAAATTATC	2000
GATGAGCGTC	GTGGTTATGC	CGATCGCGTC	ACACTACGTC	TGAACGTCGA	2050
AAACCCGAAA	CTGTGGAGCG	CCGAAATCCC	GAATCTCTAT	CGTGCAGTGG	2100
TTGAACTGCA	CACCGCCGAC	GGCACGCTGA	TTGAAGCAGA	AGCCTGCGAT	2150
GTGGTTTCC	GCGAGGTGCG	GATTGAAAAT	GGTCTGCTGC	TGCTGAACGG	2200

103

CAAGCCGTTG	CTGATTCGAG	GCGTTAACCG	TCACGAGCAT	CATCCTCTGC	2250
ATGGTCAGGT	CATGGATGAG	CAGACGATGG	TGCAGGATAT	CCTGCTGATG	2300
AAGCAGAACCA	ACTTTAACGC	CGTGCCTGT	TCGCATTATC	CGAACCATCC	2350
GCTGTGGTAC	ACGCTGTGCG	ACCGCTACGG	CCTGTATGTG	GTGGATGAAG	2400
CCAATATTGA	AACCCACGGC	ATGGTGCCAA	TGAATCGTCT	GACCGATGAT	2450
CCCGCGCTGGC	TACCGGCGAT	GAGCGAACGC	GTAACCGAA	TGGTGCAGCG	2500
CGATCGTAAT	CACCCGAGTG	TGATCATCTG	GTCGCTGGGG	AATGAATCAG	2550
GCCACGGCGC	TAATCACGAC	GCGCTGTATC	GCTGGATCAA	ATCTGTCGAT	2600
CCTTCCCGCC	CGGTGCAGTA	TGAAGGCGGC	GGAGCCGACA	CCACGGCCAC	2650
CGATATTATT	TGCCCCGATGT	ACGCGCGCGT	GGATGAAGAC	CAGCCCTTCC	2700
CGGCTGTGCC	GAAATGGTCC	ATCAAAAAAT	GGCTTCGCT	ACCTGGAGAG	2750
ACGGCGCCCGC	TGATCCTTG	CGAATACGCC	CACCGATGG	GTAACAGTCT	2800
TGGCGGTTTC	GCTAAATACT	GGCAGGGT	TCGTCACTAT	CCCCGTTTAC	2850
AGGGCGGCTT	CGTCTGGAC	TGGGTGGATC	AGTCGCTGAT	TAAATATGAT	2900
GAAAACGGCA	ACCCGTGGTC	GGCTTACGGC	GGTGATTTG	GCGATACGCC	2950
GAACGATCGC	CAGTTCTGTA	TGAACGGTCT	GGTCTTGCC	GACCGCACGC	3000
CGCATCCAGC	GCTGACGGAA	GCAAAACACC	AGCAGCAGTT	TTTCCAGTTC	3050
CGTTTATCCG	GGCAAACCAT	CGAAGTGACC	AGCGAATACC	TGTTCCGTCA	3100
TAGCGATAAC	GAGCTCCTGC	ACTGGATGGT	GGCGCTGGAT	GGTAAGCCGC	3150
TGGCAAGCGG	TGAAGTGCCT	CTGGATGTCG	CTCCACAAGG	TAAACAGTTG	3200
ATTGAACTGC	CTGAACTACC	GCAGCCGGAG	AGCGCCGGGC	AACTCTGGCT	3250
CACAGTACGC	GTAATGCAAC	CGAACGCGAC	CGCATGGTCA	GAAGCCGGGC	3300
ACATCAGCGC	CTGGCAGCAG	TGGCGTCTGG	CGGAAAACCT	CAGTGTGACG	3350
CTCCCCGCCG	CGTCCCACGC	CATCCGCAT	CTGACCACCA	GCGAAATGGA	3400
TTTTTGCATC	GAGCTGGTA	ATAAGCGTTG	GCAATTAAAC	CGCCAGTCAG	3450
GCTTTCTTTC	ACAGATGTGG	ATTGGCGATA	AAAAACAACT	GCTGACGCCG	3500

104

CTGCGCGATC AGTCACCCG TGCACCGCTG GATAACGACA TTGGCGTAAG	3550
TGAAGCGACC CGCATTGACC CTAACGCCTG GGTCGAACGC TGGAAGGCAG	3600
CGGGCCATTA CCAGGCCGAA GCAGCGTTGT TGCAGTGCAC GGCAGATACA	3650
CTTGCTGATG CGGTGCTGAT TACGACCGCT CACGCGTGGC AGCATCAGGG	3700
GAAAACCTTA TTTATCAGCC GGAAAACCTA CCGGATTGAT GGTAGTGGTC	3750
AAATGGCGAT TACCGTTGAT GTTGAAGTGG CGAGCGATAAC ACCGCATCCG	3800
GCGCGGATTG GCCTGAAC TG CCAGCTGGCG CAGGTAGCAG AGCGGGTAAA	3850
CTGGCTCGGA TTAGGGCCGC AAGAAAAC TA TCCCGACCGC CTTACTGCCG	3900
CCTGTTTGAT CCGCTGGAT CTGCCATTGT CAGACATGTA TACCCGTAC	3950
GTCTTCCCAGA GCGAAAACGG TCTGCGCTGC GGGACGCGCG AATTGAATTA	4000
TGGCCCACAC CAGTGGCGCG GCGACTTCCA GTTCAACATC AGCCGCTACA	4050
GTCAACAGCA ACTGATGGAA ACCAGCCATC GCCATCTGCT GCACGCGGAA	4100
GAAGGCACAT GGCTGAATAT CGACGGTTTC CATATGGGA TTGGTGGCGA	4150
CGACTCCTGG AGCCCGTCAG TATCGGCGGA ATTACAGCTG AGCGCCGGTC	4200
GCTACCATT A CCAGTGGTC TGGTGTCAAA AATAATAATA ACCGGGCAGG	4250
CCATGTCTGC CCGTATTCG CGTAAGGAAA TCCATTATGT ACTATTTAAA	4300
AAACACAAAC TTTTGGATGT TCGGTTTATT CTTTTCTTT TACTTTTTA	4350
TCATGGGAGC CTACTCCCCG TTTTTCCCAGA TTTGGCTACA TGACATCAAC	4400
CATATCAGCA AAAGTGATAC GGGTATTATT TTTGCCGCTA TTTCTCTGTT	4450
CTCGCTATT A TTCCAACCGC TGTTGGTCT GCTTTCTGAC AAACCTGGCC	4500
TCGACTCTAG GCGGCCGCGG GGATCCAGAC ATGATAAGAT ACATTGATGA	4550
GTTTGGACAA ACCACAACTA GAATGCAGTG AAAAAAAATGC TTTATTTGTG	4600
AAATTTGTGA TGCTATTGCT TTATTTGTAA CCATTATAAG CTGCAATAAA	4650
CAAGTTAAC A ACAACAATTG CATTCACTTT ATGTTTCAGG TTCAGGGGGA	4700
GGTGTGGAG GTTTTTCCGG ATCCTCTAGA GTCGACCTGC AGGGGCTAGA	4750
ATGGCTACGT AGATAAGTAG CATGGCGGGT TAATCATTAA CTACAAGGAA	4800

105

CCCCTAGTGA	TGGAGTTGGC	CACTCCCTCT	CTGCGCGCTC	GCTCGCTCAC	4850
TGAGGCCGGG	CGACCAAAGG	TCGCCCAGC	CCCAGGCTTT	GCCCGGGCGG	4900
CCTCAGTGAG	CGAGCGAGCG	CGCAGCTGGC	GTAATAGCGA	AGAGGCCCGC	4950
ACCGATCGCC	CTTCCCAACA	GTTGCGCAGC	CTGAATGGCG	AATGGAATT	5000
CAGACGATTG	AGCGTAAAAA	TGTAGGTATT	TCCATGAGCG	TTTTCCCTGT	5050
TGCAATGGCT	GGCGGTAATA	TTGTTCTGGA	TATTACCAAGC	AAGGCCGATA	5100
GTGGAGTTTC	TTCTACTCAG	GCAAGTGATG	TTATTACTAA	TCAAAGAAGT	5150
ATTGCGACAA	CGGTTAATT	CGGTGATGGA	CAGACTCTTT	TACTCGGTGG	5200
CCTCACTGAT	TATAAAAACA	CTTCTCAGGA	TTCTGGCGTA	CCGTTCCCTGT	5250
CTAAAATCCC	TTAATCGGC	CTCCTGTTA	GCTCCCGCTC	TGATTCTAAC	5300
GAGGAAAGCA	CGTTATACGT	GCTCGTAAA	GCAACCATAG	TACCGGCCCT	5350
GTAGCGGC	ATTAAGCGCG	GCAGGTGTGG	TGGTTACCGCG	CAGCGTGACC	5400
GCTACACTTG	CCAGCGCCCT	AGCGCCCGCT	CCTTCGCTT	TCTCCCTTC	5450
CTTTCTCGCC	ACGTTCGCCG	GCTTCGGCG	TCAAGCTCTA	AATCGGGGGC	5500
TCCCTTTAGG	GTTCCGATT	AGTGCTTAC	GGCACCTCGA	CCCCAAAAAA	5550
CTTGATTAGG	GTGATGGTTC	ACGTAGTGGG	CCATCGCCCT	GATAGACGGT	5600
TTTTCGCCCT	TTGACGTTGG	AGTCCACGTT	CTTTAATAGT	GGACTCTTGT	5650
TCCAAACTGG	AACAACACTC	AACCCTATCT	CGGTCTATT	TTTGATT	5700
TAAGGGATT	TGCCGATTTC	GGCCTATTGG	TTAAAAAAATG	AGCTGATT	5750
ACAAAAAATT	AACGCGAATT	TTAACAAAAT	ATTAACGTTT	ACAATTAAA	5800
TATTTGCTTA	TACAATCTTC	CTGTTTTGG	GGCTTTCTG	ATTATCAACC	5850
GGGGTACATA	TGATTGACAT	GCTAGTTTA	CGATTACCGT	TCATCGATT	5900
TCTTGTGTC	TCCAGACTCT	CAGGCAATGA	CCTGATAGCC	TTTGTAGAGA	5950
CCTCTCAAAA	ATAGCTACCC	TCTCCGGCAT	GAATTATCA	GCTAGAACGG	6000
TTGAATATCA	TATTGATGGT	GATTTGACTG	TCTCCGGCCT	TTCTCACCCG	6050
TTTGAATCTT	TACCTACACA	TTACTCAGGC	ATTGCATT	AAATATATGA	6100

106

GGGTTCTAAA	AATTTTATC	CTTGCCTTGA	AATAAAGGCT	TCTCCCGCAA	6150
AAGTATTACA	GGGTCTATAAT	TTTTTGGTA	CAACCGATTT	AGCTTTATGC	6200
TCTGAGGCTT	TATTGCTTAA	TTTGCTAAT	TCTTGCCTT	GCCTGTATGA	6250
TTTATTGGAT	GTTGGAATTC	CTGATGCGGT	ATTTTCTCCT	TACGCATCTG	6300
TGCGGTATT	CACACCGCAT	ATGGTGCACT	CTCAGTACAA	TCTGCTCTGA	6350
TGCCGCATAG	TTAACGCCAGC	CCCGACACCC	GCCAACACCC	GCTGACGCGC	6400
CCTGACGGGC	TTGTCTGCTC	CCGGCATCCG	CTTACAGACA	AGCTGTGACC	6450
GTCTCCGGGA	GCTGCATGTG	TCAGAGGTTT	TCACCGTCAT	CACCGAAACCG	6500
CGCGAGACGA	AAGGGCCTCG	TGATAACGCCT	ATTTTTATAG	GTAAATGTCA	6550
TGATAATAAT	GGTTTCTTAG	ACGTCAGGTG	GCACCTTCG	GGGAAATGTG	6600
CGCGGAACCC	CTATTGTTT	ATTTTTCTAA	ATACATTCAA	ATATGTATCC	6650
GCTCATGAGA	CAATAACCCT	GATAAATGCT	TCAATAATAT	TGAAAAAGGA	6700
AGAGTATGAG	TATTCAACAT	TTCCGTGTCG	CCCTTATTCC	CTTTTTGCG	6750
GCATTTGCC	TTCCTGTTT	TGCTCACCCA	GAAACGCTGG	TGAAAGTAAA	6800
AGATGCTGAA	GATCAGTTGG	GTGCACGGAGT	GGGTTACATC	GAACGGATC	6850
TCAACAGCGG	TAAGATCCTT	GAGAGTTTC	GCCCCGAAGA	ACGTTTCCA	6900
ATGATGAGCA	CTTTTAAAGT	TCTGCTATGT	GGCGCGGTAT	TATCCCGTAT	6950
TGACGCCGGG	CAAGAGAAC	TCGGTCGCCG	CATACACTAT	TCTCAGAATG	7000
ACTGGTTGA	GTACTCACCA	GTCACAGAAA	AGCATCTTAC	GGATGGCATG	7050
ACAGTAAGAG	AATTATGCAG	TGCTGCCATA	ACCATGAGTG	ATAACACTGC	7100
GGCCAACCTTA	CTTCTGACAA	CGATCGGAGG	ACCGAAGGAG	CTAACCGCTT	7150
TTTGCACAA	CATGGGGGAT	CATGTAACTC	GCCTTGATCG	TTGGGAACCG	7200
GAGCTGAATG	AAGCCATACC	AAACGACGAG	CGTGACACCA	CGATGCCTGT	7250
AGCAATGGCA	ACAACGTTGC	GCAAACATT	AACTGGCGAA	CTACTTACTC	7300
TAGCTTCCCG	GCAACAATTA	ATAGACTGGA	TGGAGGCGGA	TAAAGTTGCA	7350
GGACCACTTC	TGCGCTCGGC	CCTTCCGGCT	GGCTGGTTA	TTGCTGATAA	7400

107

ATCTGGAGCC	GGTGAGCGTG	GGTCTCGCGG	TATCATTGCA	GCACTGGGGC	7450
CAGATGGTAA	GCCCTCCCGT	ATCGTAGTTA	TCTACACGAC	GGGGAGTCAG	7500
GCAACTATGG	ATGAACGAAA	TAGACAGATC	GCTGAGATAG	GTGCCTCACT	7550
GATTAAGCAT	TGGTAACTGT	CAGACCAAGT	TTACTCATAT	ATACTTTAGA	7600
TTGATTTAAA	ACTTCATTTC	TAATTTAAAA	GGATCTAGGT	GAAGATCCTT	7650
TTTGATAATC	TCATGACCAA	AATCCCTTAA	CGTGAGTTTT	CGTCCACTG	7700
AGCGTCAGAC	CCCCTAGAAA	AGATCAAAGG	ATCTTCTTGA	GATCCTTTTT	7750
TTCTGCGCGT	AATCTGCTGC	TTGCAAACAA	AAAAACCACC	GCTACCAGCG	7800
GTGGTTTGT	TGCCGGATCA	AGAGCTACCA	ACTCTTTTC	CGAAGGTAAC	7850
TGGCTTCAGC	AGAGCGCAGA	TACCAAATAC	TGTCTTCTA	GTGTAGCCGT	7900
AGTTAGGCCA	CCACTTCAAG	AACTCTGTAG	CACCGCCTAC	ATACCTCGCT	7950
CTGCTAATCC	TGTTACCACT	GGCTGCTGCC	AGTGGCGATA	AGTCGTGTCT	8000
TACCGGGTTG	GACTCAAGAC	GATA GTTACC	GGATAAGGCG	CAGCGGTCGG	8050
GCTGAACGGG	GGGTTCGTGC	ACACAGCCCA	GCTTGGAGCG	AACGACCTAC	8100
ACCGAACTGA	GATA CCTACA	GCGTGAGCTA	TGAGAAAGCG	CCACGCTTCC	8150
CGAAGGGAGA	AAGGCAGACA	GGTATCCGGT	AAGCGGCAGG	GTCCGAACAG	8200
GAGAGCGCAC	GAGGGAGCTT	CCAGGGGGAA	ACGCCTGGTA	TCTTTATAGT	8250
CCTGTCGGGT	TTCGCCACCT	CTGACTTGAG	CGTCGATTTC	TGTGATGCTC	8300
GTCAGGGGGG	CGGAGCCTAT	GGAAAAACGC	CAGCAACGCG	GCCTTTTAC	8350
GGTTCCCTGGC	CTTTTGCTGG	CCTTTGCTC	ACATGTTCTT	TCCTGCGTTA	8400
TCCCCCTGATT	CTGTGGATAA	CCGTATTACC	GCCTTGAGT	GAGCTGATAC	8450
CGCTCGCCGC	AGCCGAACGA	CCGAGCGCAG	CGAGTCAGTG	AGCGAGGAAG	8500
CGGAAGAGC					8509

## (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8299 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: not relevant

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCCAATACG	CAAACCGCCT	CTCCCCGCGC	GTTGGCCGAT	TCATTAATGC	50
AGCTGCGCGC	TCGCTCGCTC	ACTGAGGCCG	CCCGGGCAAA	GCCCGGGCGT	100
CGGGCGACCT	TTGGTCGCCC	GGCCTCAGTG	AGCGAGCGAG	CGCGCAGAGA	150
GGGAGTGGCC	AACTCCATCA	CTAGGGTTC	CTTGTAGTTA	ATGATTAACC	200
CGCCATGCTA	CTTATCTACA	TCATCGATGA	ATTGAGCCTT	GCATGCCTGC	250
AGGTCGTTAC	ATAACTTACG	GTAAATGGCC	CGCCTGGCTG	ACCGCCCAAC	300
GACCCCCGCC	CATTGACGTC	AATAATGACG	TATGTTCCCA	TAGTAACGCC	350
AATAGGGACT	TTCCATTGAC	GTCAATGGGT	GGAGTATTAA	CGGTAAACTG	400
CCCACTTGGC	AGTACATCAA	GTGTATCATA	TGCCAAGTAC	GCCCCCTATT	450
GACGTCAATG	ACGGTAAATG	GCCCCCTGG	CATTATGCC	AGTACATGAC	500
CTTATGGGAC	TTTCCTACTT	GGCAGTACAT	CTACGTATTA	GTCATCGCTA	550
TTACCATGGT	GATGCGGTTT	TGGCAGTACA	TCAATGGCG	TGGATAGCGG	600
TTTGACTCAC	GGGGATTCC	AAGTCTCCAC	CCCATTGACG	TCAATGGGAG	650
TTTGTGTTGG	CACCAAAATC	AACGGGACTT	TCCAAATGT	CGTAACAACT	700
CCGCCCCATT	GACGCAAATG	GGCGGTAGGC	GTGTACGGTG	GGAGGTCTAT	750
ATAAGCAGAG	CTCGTTAGT	GAACCGTCAG	ATCGCCTGGA	GACGCCATCC	800
ACGCTGTTT	GACCTCCATA	GAAGACACCG	GGACCGATCC	AGCCTCCGG	850
CTCTAGAGGA	TCCGGTACTC	GACCCGAGCT	CGGATCCACT	AGTAACGGCC	900
GCCAGTGTGC	TGGAATTCTG	CACTCCAGGC	TGCCCCGGTT	TGCATGCTGC	950
TGCTGCTGCT	GCTGCTGGGC	CTGAGGCTAC	AGCTCTCCCT	GGGCATCATC	1000

109

CTAGTTGAGG	AGGAGAACCC	GGACTTCTGG	AACCGCGAGG	CAGCCGAGGC	1050
CCTGGGTGCC	GCCAAGAACG	TGCAGCCTGC	ACAGACAGCC	GCCAAGAACCC	1100
TCATCATCTT	CCTGGGCGAT	GGGATGGGGG	TGTCTACGGT	GACAGCTGCC	1150
AGGATCCTAA	AAGGGCAGAA	GAAGGACAAA	CTGGGGCCTG	AGATACCCCT	1200
GGCCATGGAC	CGCTTCCCCT	ATGTGGCTCT	GTCCAAGACA	TACAATGTAG	1250
ACAAACATGT	GCCAGACAGT	GGAGCCACAG	CCACGGCCTA	CCTGTGCGGG	1300
GTCAAGGGCA	ACTTCCAGAC	CATTGGCTTG	AGTGCAGCCG	CCCGCTTTAA	1350
CCAGTGCAAC	ACGACACGCG	GCAACGAGGT	CATCTCCGTG	ATGAATCGGG	1400
CCAAGAAAGC	AGGGAAGTCA	GTGGGAGTGG	TAACCACCAC	ACGAGTGCAG	1450
CACGCCCTCGC	CAGCCGGCAC	CTACGCCAC	ACGGTGAACC	GCAACTGGTA	1500
CTCGGACGCC	GACGTGCCTG	CCTCGGCCCCG	CCAGGAGGGG	TGCCAGGACA	1550
TCGCTACGCA	GCTCATCTCC	AACATGGACA	TTGATGTGAT	CCTAGGTGGA	1600
GGCCGAAAGT	ACATGTTTCG	CATGGGAACC	CCAGACCCCTG	AGTACCCAGA	1650
TGACTACAGC	CAAGGTGGGA	CCAGGCTGGA	CGGGAAGAAT	CTGGTGCAGG	1700
AATGGCTCGG	CGAACGCCAG	GGTGCCCCGT	ACGTGTGGAA	CCGCACTGAG	1750
CTCATGCAGG	CTTCCCTGGA	CCCGTCTGTG	ACCCATCTCA	TGGGTCTCTT	1800
TGAGCCTGGA	GACATGAAAT	ACGAGATCCA	CCGAGACTCC	ACACTGGACC	1850
CCTCCCTGAT	GGAGATGACA	GAGGCTGCC	TGCGCCTGCT	GAGCAGACAC	1900
CCCCCGGGCT	TCTTCCTCTT	CGTGGAGGGT	GGTCGCGATCG	ACCATGGTCA	1950
TCATGAAAGC	AGGGCTTACC	GGGCACTGAC	TGAGACGATC	ATGTTCGACG	2000
ACGCCATTGA	GAGGGCGGGC	CAGCTCACCA	GCGAGGGAGGA	CACGCTGAGC	2050
CTCGTCACTG	CCGACCACTC	CCACGTCTTC	TCCTTCGGAG	GCTACCCCT	2100
GCGAGGGAGC	TCCTTCATCG	GGCTGGCCGC	TGGCAAGGCC	CGGGACAGGA	2150
AGGCCTACAC	GGTCCTCCTA	TACGGAAACG	GTCCAGGCTA	TGTGCTCAAG	2200
GACGGCGCCC	GGCCGGATGT	TACCGAGAGC	GAGAGCGGGG	GCCCCGAGTA	2250
TCGGCAGCAG	TCAGCAGTGC	CCCTGGACGA	AGAGACCCAC	GCAGGGCGAGG	2300

110

ACGTGGCGGT	GTTCGCGCGC	GGCCCGCAGG	CGCACCTGGT	TCACGGCGTG	2350
CAGGAGCAGA	CCTTCATAGC	GCACGTATG	GCCTTCGCCG	CCTGCCTGGA	2400
GCCCTACACC	GCCTGCGACC	TGGCGCCCCC	CGCCGGCACC	ACCGACGCCG	2450
CGCACCCGGG	GCGGTCCGTG	GTCCCCGCGT	TGCTTCCTCT	GCTGGCCGGG	2500
ACCTGCTGC	TGCTGGAGAC	GGCCACTGCT	CCCTGAGTGT	CCCGTCCCTG	2550
GGGCTCCTGC	TTCCCCATCC	CGGAGTTCTC	CTGCTCCCCA	CCTCCTGTGCG	2600
TCCTGCCTGG	CCTCCAGCCC	GAGTCGTAT	CCCCGGAGTC	CCTATACAGA	2650
GGTCCTGCCA	TGGAACCTTC	CCCTCCCCGT	GCGCTCTGGG	GACTGAGCCC	2700
ATGACACCAA	ACCTGCCCC	TGGCTGCTCT	CGGACTCCCT	ACCCCAACCC	2750
CAGGGACTGC	AGGTTGTGCC	CTGTGGCTGC	CTGCACCCCA	GGAAAGGAGG	2800
GGGCTCAGGC	CATCCAGCCA	CCACCTACAG	CCCAGTGGGG	TCGAGACAGA	2850
TGGTCAGTCT	GGAGGATGAC	GTGGCGTGAA	GCTGGCCGCG	GGGATCCAGA	2900
CATGATAAGA	TACATTGATG	AGTTTGGACA	AACCACAACT	AGAATGCAGT	2950
GAAAAAAATG	CTTTATTTGT	GAAATTGTG	ATGCTATTGC	TTTATTTGTA	3000
ACCATTATAA	GCTGCAATAA	ACAAGTTAAC	AACAACAATT	GCATTCATTT	3050
TATGTTTCAG	GTTCAGGGGG	AGGTGTGGGA	GGTTTTTCG	GATCCTCTAG	3100
AGTCGACTCT	AGANNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3150
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3200
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3250
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3300
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	3350
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNGGATCCC	3400
CATGACTACG	TCCGGCGTTC	CATTTGGCAT	GACACTACGA	CCAACACGAT	3450
CTCGGTTGTC	TCGGCGCACT	CCGTACAGTA	GGGATCGTCT	ACCTCCTTTT	3500
GAGACAGAAA	CCCGCGCTAC	CATACTGGAG	GATCATCCGC	TGCTGCCCGA	3550
ATGTAACACT	TTGACAATGC	ACAACGTGAG	TTACGTGCGA	GGTCTTCCCT	3600

111

GCAGTGTGGG ATTTACGCTG ATTCAAGGAAT GGGTTGTTCC CTGGGATATG	3650
GTTCTAACGC GGGAGGAGCT TGTAATCCTG AGGAAGTGTG TGCACGTGTG	3700
CCTGTGTTGT GCCAACATTG ATATCATGAC GAGCATGATG ATCCATGGTT	3750
ACGAGTCCTG GGCTCTCCAC TGTCAATTGTT CCAGTCCCGG TTCCCTGCAG	3800
TGTATAGCCC GCGGGCAGGT TTTGGCCAGC TGTTTAGGA TGTTGGTGG	3850
TGGCGCCATG TTTAACATCAGA GGTTTATATG GTACCGGGAG GTGGTGAATT	3900
ACAACATGCC AAAAGAGGTA ATGTTTATGT CCAGCGTGT TATGAGGGGT	3950
CGCCACTTAA TCTACCTGCG CTTGTGGTAT GATGGCCACG TGGGTTCTGT	4000
GGTCCCCGCC ATGAGCTTTG GATACAGCGC CTTGCACTGT GGGATTTGA	4050
ACAATATTGT GGTGCTGTGC TGCAGTTACT GTGCTGATTT AAGTGAGATC	4100
AGGGTGCCT GCTGTGCCCG GAGGACAAGG CGCCTTATGC TGCGGGCGGT	4150
GCGAATCATC GCTGAGGAGA CCACTGCCAT GTTGTATTCC TGCAGGACGG	4200
AGCGGCGGCG GCAGCAGTTT ATTGCGCGC TGCTGCAGCA CCACCGCCCT	4250
ATCCTGATGC ACGATTATGA CTCTACCCCC ATGTAGGGAT CCCCCATCACT	4300
AGTGCAGGCC CGGGGATCCA GACATGATAA GATACATTGA TGAGTTGG	4350
CAAACACAA CTAGAATGCA GTGAAAAAAA TGCTTTATTT GTGAAATTTG	4400
TGATGCTATT GCTTTATTTG TAACCATTAT AAGCTGCAAT AAACAAGTTA	4450
ACAACACAA TTGCATTCA TTTATGTTTC AGGTTCAGGG GGAGGTGTGG	4500
GAGGTTTTT CGGATCCTCT AGAGTCGACC TGCAAGGCATG CAAGCTGTAG	4550
ATAAGTAGCA TGGCGGGTTA ATCATTAACt ACAAGGAACC CCTAGTGATG	4600
GAGTTGGCCA CTCCCTCTCT GCGCGCTCGC TCGCTCACTG AGGCCGGCG	4650
ACCAAAGGTC GCCCGACGCC CGGGCTTGC CCGGGCGGCC TCAGTGAGCG	4700
AGCGAGCGCG CAGCTGGCGT AATAGCGAAG AGGCCCGCAC CGATGCCCT	4750
TCCCAACAGT TGCGCAGCCT GAATGGCGAA TGGAANTTCC AGACGATTGA	4800
GCGTCAAAAT GTAGGTATTT CCATGAGCGT TTTCTGTGTT GCAATGGCTG	4850
GCGGTAATAT TGTTCTGGAT ATTACCAAGCA AGGCCGATAG TTTGAGTTCT	4900

112

TCTACTCAGG	CAAGTGATGT	TATTACTAAT	CAAAGAAGTA	TTGCGACAAC	4950
GGTTAATTG	CGTGATGGAC	AGACTCTTT	ACTCGGTGGC	CTCACTGATT	5000
ATAAAAACAC	TTCTCAGGAT	TCTGGCGTAC	CGTTCCGTGC	TAAAATCCCT	5050
TTAATCGGCC	TCCTGTTAG	CTCCCGCTCT	GATTCTAACG	AGGAAAGCAC	5100
GTTATACGTG	CTCGTCAAAG	CAACCATACT	ACGCGCCCTG	TAGCGGCGCA	5150
TTAAGCGCGG	CGGGTGTGGT	GGTTACCGCGC	AGCGTGACCG	CTACACTTGC	5200
CAGCGCCCTA	GCGCCCGCTC	CTTTCGCTTT	CTTCCCTTCC	TTTCTGCCA	5250
CGTTCGCCGG	CTTTCCCCGT	CAAGCTCTAA	ATCGGGGGCT	CCCTTTAGGG	5300
TTCCGATTAA	GTGCTTTACG	GCACCTCGAC	CCCAAAAAAC	TTGATTAGGG	5350
TGATGGTTCA	CGTAGTGGGC	CATCGCCCTG	ATAGACGGTT	TTTCGCCCTT	5400
TGACGTTGGA	GTCCACGTTC	TTTAATAGTG	GACTCTTGT	CCAAACTGGA	5450
ACAACACTCA	ACCCTATCTC	GGTCTATTCT	TTTGATTTAT	AAGGGATTTT	5500
GCCGATTTCG	GCCTATTGGT	AAAAAAATGA	GCTGATTTAA	AAAAAAATTAA	5550
ACGCGAATT	TAACAAAATA	TTAACGTTA	CAATTAAAT	ATTTGCTTAT	5600
ACAATCTTCC	TGTTTTGGG	GCTTTCTGA	TTATCAACCG	GGGTACATAT	5650
GATTGACATG	CTAGTTTAC	GATTACCGTT	CATCGATTCT	CTTGTGTTGCT	5700
CCAGACTCTC	AGGCAATGAC	CTGATAGCCT	TTGTAGAGAC	CTCTCAAAAA	5750
TAGCTACCCT	CTCCGGCATG	AATTTATCAG	CTAGAACGGT	TGAATATCAT	5800
ATTGATGGTG	ATTTGACTGT	CTCCGGCCTT	TCTCACCCGT	TTGAATCTTT	5850
ACCTACACAT	TACTCAGGCA	TTGCATTTAA	AATATATGAG	GGTTCTAAAA	5900
ATTTTTATCC	TTGCGTTGAA	ATAAAGGCTT	CTCCCGCAAA	AGTATTACAG	5950
GGTCATAATG	TTTTGGTAC	AACCGATTTA	GCTTTATGCT	CTGAGGCTTT	6000
ATTGCTTAAT	TTTGCTAATT	CTTGCCTTG	CCTGTATGAT	TTATTGGATG	6050
TTGGAANTTC	CTGATGCGGT	ATTTCTCCT	TACGCATCTG	TGCGGTATTT	6100
CACACCGCAT	ATGGTGCACT	CTCAGTACAA	TCTGCTCTGA	TGCCGCATAG	6150
TTAACCCAGC	CCCGACACCCC	GCCAACACCCC	GCTGACGCGC	CCTGACGGGC	6200

113

TTGTCTGCTC	CCGGCATCCG	CTTACAGACA	AGCTGTGACC	GTCTCCGGGA	6250
GCTGCATGTG	TCAGAGGTTT	TCACCGTCAT	CACCGAAACG	CGCGAGACGA	6300
AAGGGCCTCG	TGATACGCCT	ATTTTATAG	GTAAATGTCA	TGATAATAAT	6350
GGTTTCTTAG	ACGTCAGGTG	GCACCTTCG	GGGAAATGTG	CGCGGAACCC	6400
CTATTTGTTT	ATTTTCTAA	ATACATTCAA	ATATGTATCC	GCTCATGAGA	6450
CAATAACCCT	GATAAATGCT	TCAATAATAT	TGAAAAAGGA	AGAGTATGAG	6500
TATTCAACAT	TTCCGTGTCG	CCCTTATTCC	CTTTTTGCG	GCATTTGCC	6550
TTCCTGTTT	TGCTCACCCA	GAAACGCTGG	TGAAAGTAAA	AGATGCTGAA	6600
GATCAGTTGG	GTGCACGAGT	GGGTTACATC	GAACGGATC	TCAACAGCGG	6650
TAAGATCCTT	GAGAGTTTC	GCCCCGAAGA	ACGTTTCCA	ATGATGAGCA	6700
CTTTTAAAGT	TCTGCTATGT	GGCGCGGTAT	TATCCCGTAT	TGACGCCGGG	6750
CAAGAGCAAC	TCGGTCGCCG	CATACACTAT	TCTCAGAATG	ACTTGGTTGA	6800
GTACTCACCA	GTCACAGAAA	AGCATCTTAC	GGATGGCATG	ACAGTAAGAG	6850
AATTATGCAG	TGCTGCCATA	ACCATGAGTG	ATAACACTGC	GGCCAACCTTA	6900
CTTCTGACAA	CGATCGGAGG	ACCGAAGGAG	CTAACCGCTT	TTTGCACAA	6950
CATGGGGGAT	CATGTAACTC	GCCTTGATCG	TTGGGAACCG	GAGCTGAATG	7000
AAGCCATACC	AAACGACGAG	CGTGACACCA	CGATGCCTGT	AGCAATGGCA	7050
ACAACGTTGC	GCAAACATT	AACTGGCGAA	CTACTTACTC	TAGCTTCCCG	7100
GCAACAATTA	ATAGACTGGA	TGGAGGCGGA	TAAAGTTGCA	GGACCACTTC	7150
TGCGCTCGGC	CCTTCCGGCT	GGCTGGTTA	TTGCTGATAA	ATCTGGAGCC	7200
GGTGAGCGTG	GGTCTCGCGG	TATCATTGCA	GCACGGGGC	CAGATGGTAA	7250
GCCCTCCCGT	ATCGTAGTTA	TCTACACGAC	GGGGAGTCAG	GCAACTATGG	7300
ATGAACGAAA	TAGACAGATC	GCTGAGATAG	GTGCCTCACT	GATTAAGCAT	7350
TGGTAACTGT	CAGACCAAGT	TTACTCATAT	ATACTTTAGA	TTGATTAAA	7400
ACTTCATTTT	TAATTAAAAA	GGATCTAGGT	GAAGATCCTT	TTTGATAATC	7450
TCATGACCAA	AATCCCTTAA	CGTGAGTTT	CGTTCCACTG	AGCGTCAGAC	7500

114

CCCGTAGAAA AGATCAAAGG ATCTTCTTGA GATCCTTTT TTCTGCGCGT	7550
AATCTGCTGC TTGCAAACAA AAAAACCAACC GCTACCAGCG GTGGTTTGT	7600
TGCCGGATCA AGAGCTACCA ACTCTTTTC CGAAGGTAAC TGGCTTCAGC	7650
AGAGCGCAGA TACCAAATAC TGTCTTCTA GTGTAGCCGT AGTTAGGCCA	7700
CCACTTCAAG AACTCTGTAG CACCGCTAC ATACCTCGCT CTGCTAATCC	7750
TGTTACCAGT GGCTGCTGCC AGTGGCGATA AGTCGTGTCT TACCGGGTTG	7800
GACTCAAGAC GATAAGTACC GGATAAGGCG CAGCGGTGG GCTGAACGGG	7850
GGGTTCGTGC ACACAGCCC A GCTTGGAGCG AACGACCTAC ACCGAACGT	7900
GATACTACA GCGTGAGCTA TGAGAAAGCG CCACGCTTCC CGAAGGGAGA	7950
AAGGCAGACA GGTATCCGGT AAGCGGCAGG GTCGGAACAG GAGAGCGCAC	8000
GAGGGAGCTT CCAGGGGGAA ACGCCTGGTA TCTTTATAGT CCTGTCGGGT	8050
TTCGCCACCT CTGACTTGAG CGTCGATTTT TGTGATGCTC GTCAGGGGG	8100
CGGAGCCTAT GGAAAAACGC CAGCAACGCG GCCTTTTAC GGTCCTGGC	8150
CTTTTGCTGG CCTTTGCTC ACATGTTCTT TCCTGCGTTA TCCCCTGATT	8200
CTGTGGATAA CCGTATTACC GCCTTGAGT GAGCTGATAC CGCTCGCCGC	8250
AGCCGAACGA CCGAGCGCAG CGAGTCAGTG AGCGAGGAAG CGGAAGAGC	8299

## WHAT IS CLAIMED IS:

1. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell *ex vivo* comprising the steps of providing a recombinant adeno-associated virus comprising: (a) the DNA of at least a portion of the genome of an adeno-associated virus which portion is capable of transducing a selected gene into a target cell in the absence of cell division; and (b) a selected gene operatively linked to regulatory sequences directing its expression, said gene flanked by the DNA of (a) and capable of expression in the target cell; infecting said target cells with said recombinant adeno-associated virus; contacting said infected cells with an agent which facilitates the conversion of said ss recombinant virus to its double stranded form, wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus into said target cell.
2. The method according to claim 1 wherein said agent is a helper virus comprising a selected gene encoding a polypeptide which can enhance the conversion of said single-stranded recombinant virus to double-stranded recombinant virus or functional fragment thereof, said helper virus capable of transducing said selected gene into the target cell in the absence of cell division.
3. The method according to claim 2 wherein said contacting step comprises co-infecting said target cell with said helper virus.
4. The method according to claim 3 wherein said helper virus is an adenovirus.

5. The method according to claim 2 wherein said selected gene is the adenovirus E4 gene or a functional fragment thereof.

6. The method according to claim 2 wherein said adenovirus contains two selected genes or functional fragments thereof, said genes being the adenovirus E4 gene and E1 gene.

7. The method according to claim 5 or 6 wherein said functional fragment of said E4 gene comprises the open reading frame 6 of E4.

8. The method according to claim 1 wherein said recombinant adeno-associated virus further comprises a second selected gene operatively linked to regulatory sequences capable of directing expression of said second gene, said second gene capable of facilitating the conversion of said single stranded recombinant virus to its double stranded form upon expression, and capable of co-expression with said first selected gene in the target cell, whereby the second gene product is expressed in the target cell, wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus in said target cell.

9. The method according to claim 8 wherein the regulatory sequences directing expression of said second gene comprise an inducible promoter, and wherein expression of said second gene occurs in the presence of an inducing agent.

10. A recombinant adeno-associated virus comprising  
(a) the DNA of at least a portion of the genome of an  
adeno-associated virus which portion is capable of  
transducing at least two selected genes or functional  
fragments thereof into a target cell in the absence of  
cell division; (b) a first selected gene operatively  
linked to regulatory sequences directing its expression,  
(c) a second selected gene operatively linked to  
regulatory sequences capable of directing expression of  
said second gene, said second gene capable of  
facilitating the conversion of said single stranded  
recombinant virus to its double stranded form upon  
expression, said first and said second genes flanked by  
the DNA of (a), said first and second gene capable of co-  
expression in the target cell.

11. The recombinant virus according to claim 10  
wherein said second gene is selected from the group  
consisting of an adenovirus E4 gene, ORF6 of E4 and a  
functional fragment thereof.

12. The recombinant virus according to claim 10  
wherein said second gene is the adenovirus E1 gene or a  
functional fragment thereof.

13. The recombinant virus according to claim 10  
wherein the regulatory sequences directing expression of  
said second gene comprise an inducible promoter, and  
wherein expression of said second gene occurs in the  
presence of an inducing agent.

14. The recombinant virus according to claim 10 further comprising (d) an additional selected gene operatively linked to regulatory sequences capable of directing expression of said gene, said additional gene and said second gene capable of jointly facilitating the conversion of said single stranded recombinant virus to its double stranded form upon expression of both said second and additional genes, said first, second and additional genes flanked by the DNA of (a), and capable of co-expression in the target cell.

15. The recombinant virus according to claim 14 wherein said second gene is the adenovirus E4 gene or a functional fragment thereof and said additional gene is the adenovirus E1 gene or a functional fragment thereof.

16. The recombinant virus according to claim 15 wherein said functional fragment of E4 is the ORF6 sequence.

17. The recombinant virus according to claim 15 wherein the regulatory sequences directing expression of said additional gene comprise an inducible promoter, and wherein expression of said additional gene occurs in the presence of an inducing agent.

18. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell comprising the steps of: providing a recombinant adeno-associated virus of claim 10 through 16; infecting said target cells with said recombinant virus; and culturing said infected cells under conditions which enable expression of said selected genes in said target cell,

wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus in said target cell.

19. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell comprising the steps of: providing a recombinant adeno-associated virus of claim 17; infecting said target cells with said recombinant virus; and contacting said target cell with an inducing agent which induces the expression of said second or additional gene in said target cell, wherein said conversion occurs in said target cell, resulting in enhanced transduction of said recombinant virus in said target cell.

20. A pharmaceutical composition comprising a recombinant adeno-associated virus and an agent which facilitates the conversion of said ss recombinant virus to its double stranded form in a target cell.

21. The composition according to claim 20 wherein said virus is selected from the group consisting of the recombinant viruses of claims 10 - 16.

22. The composition according to claim 20 wherein said virus is the recombinant virus of claim 17 and further comprising an inducing agent which induces the expression of said second or additional gene in said recombinant virus.

23. A mammalian cell transduced with the recombinant adeno-associated viruses of claims 10-17.

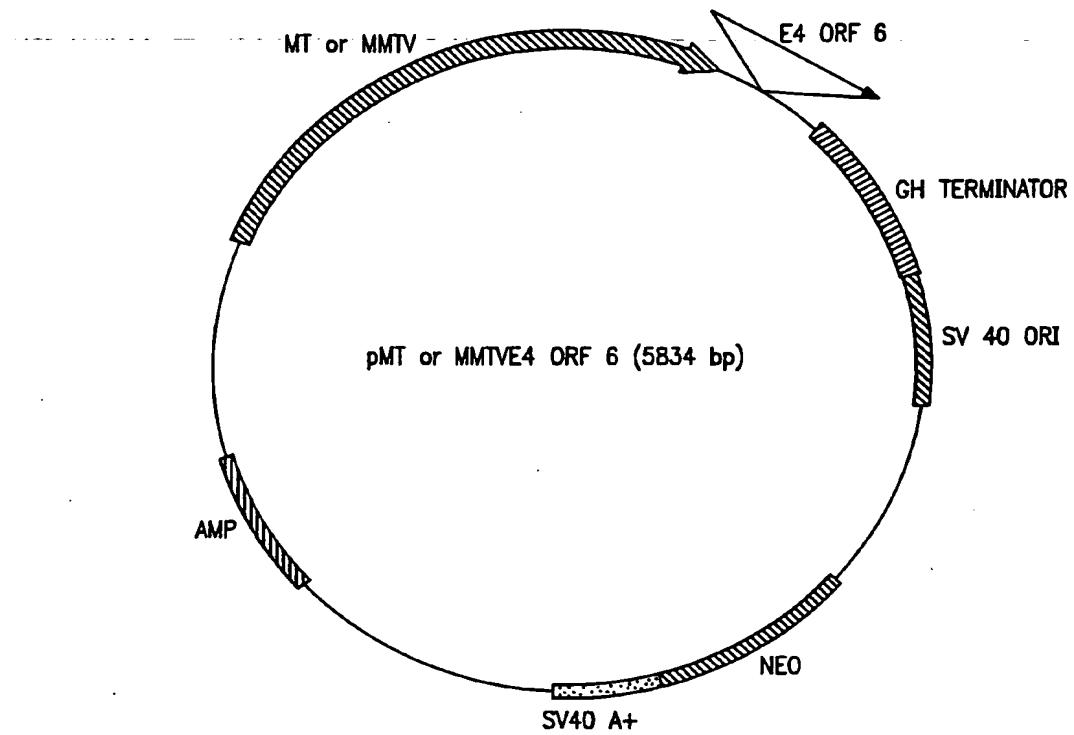
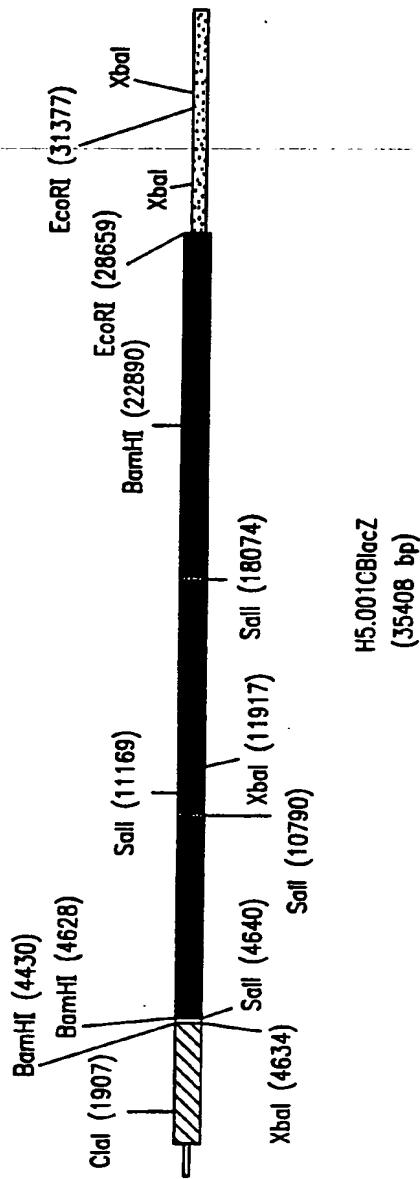
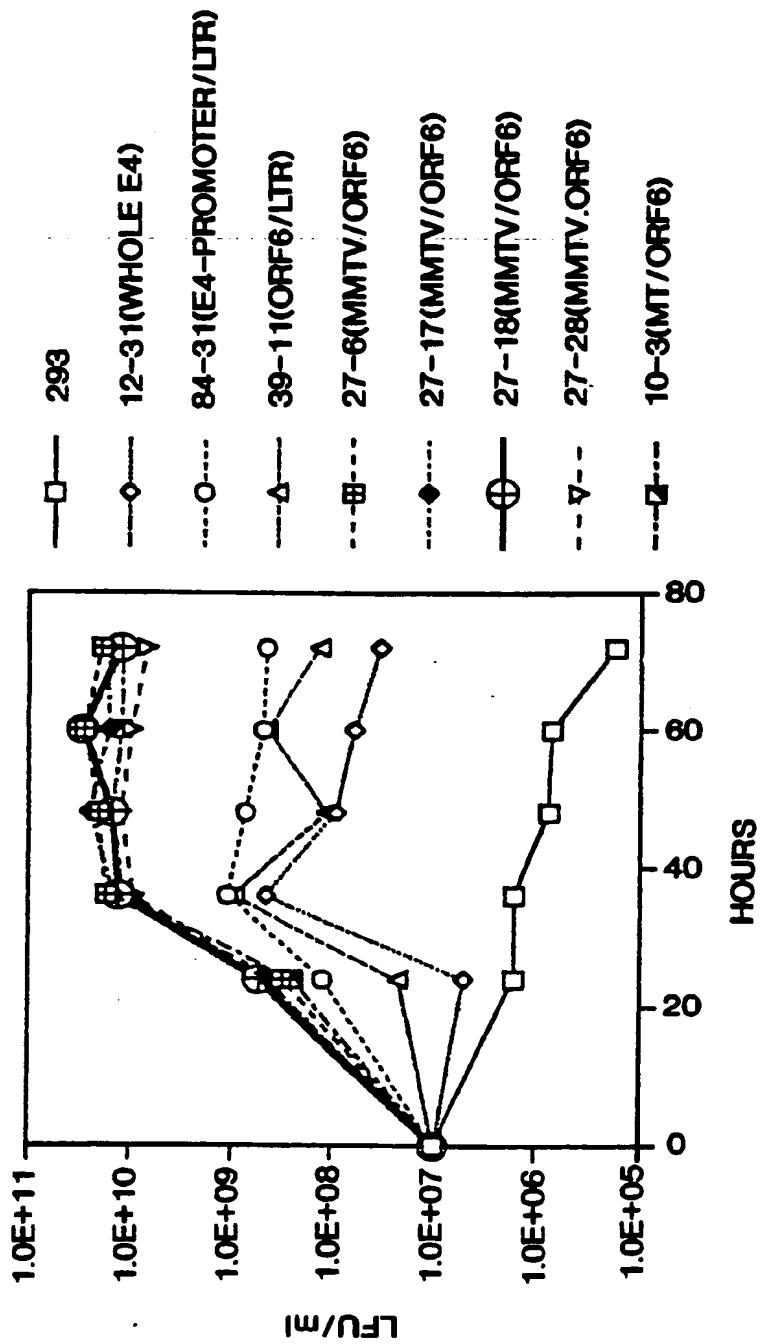


FIG. 1



**FIG. 2**

FIG. 3



4 / 11

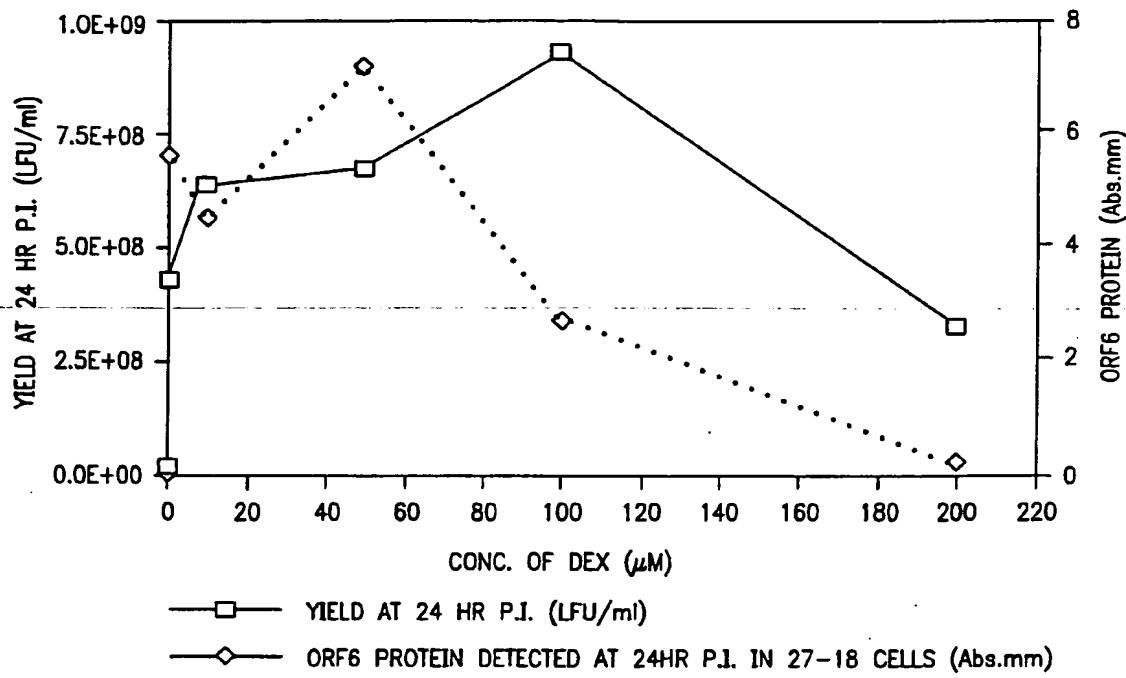


FIG. 4A

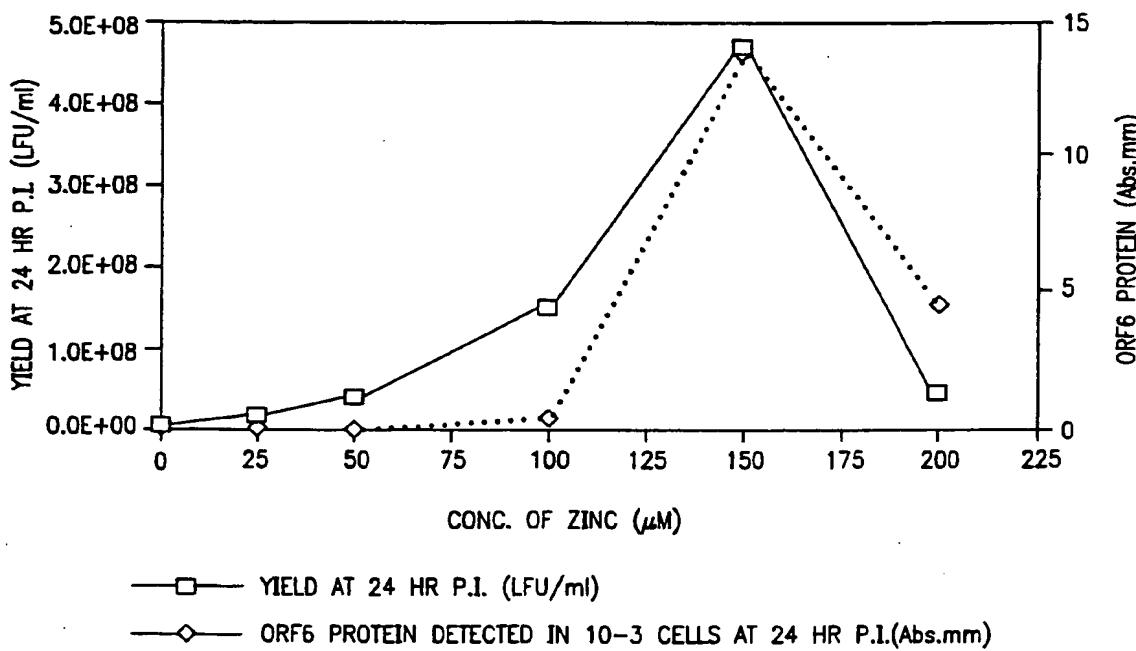


FIG. 4B

SUBSTITUTE SHEET (RULE 26)

5/11

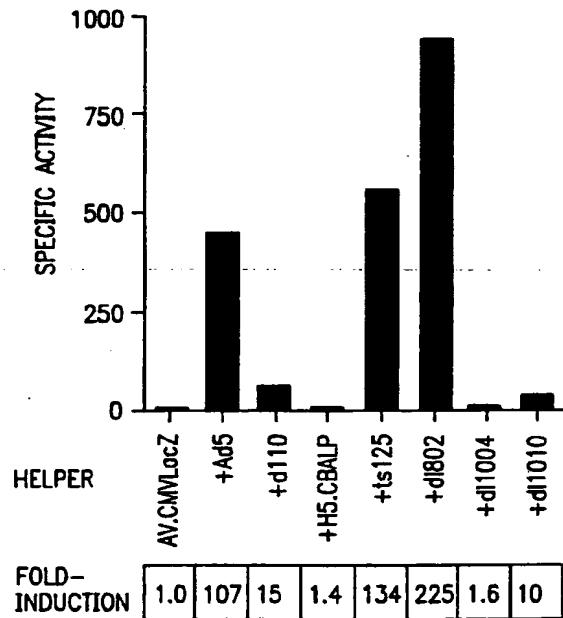


FIG. 5A

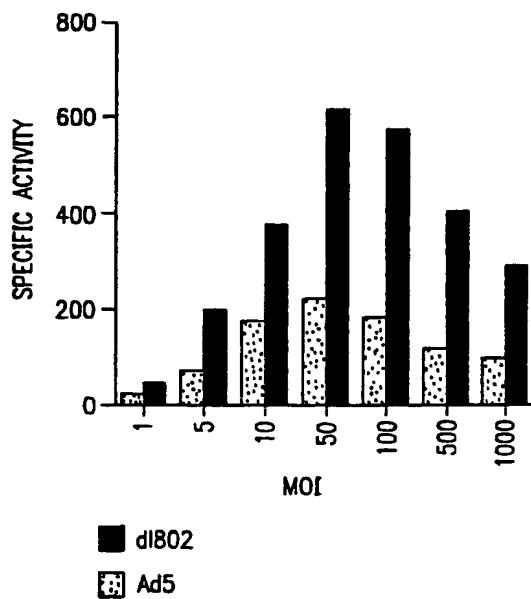


FIG. 5B

6/11

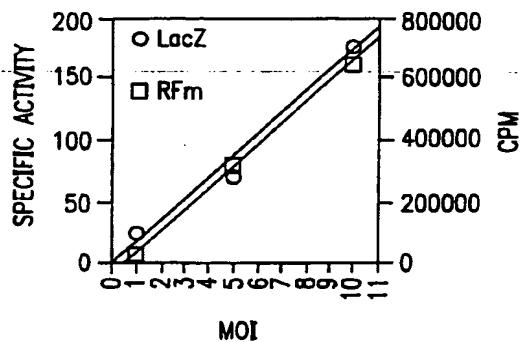


FIG. 6A

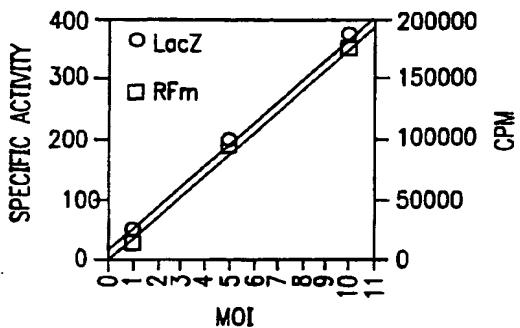


FIG. 6B

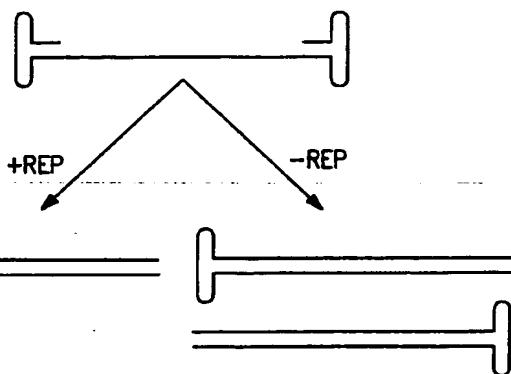
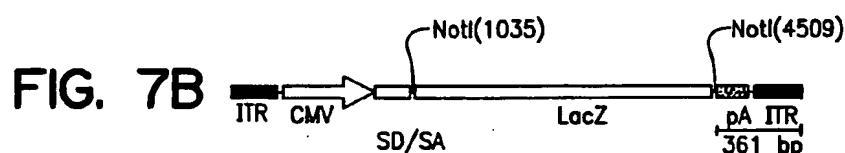
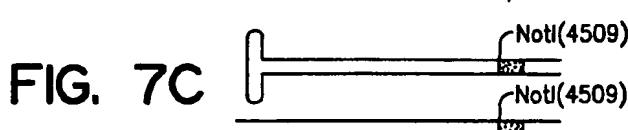
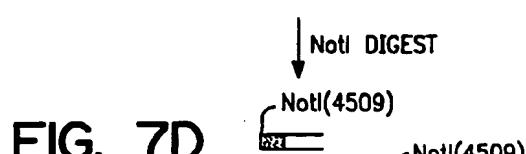
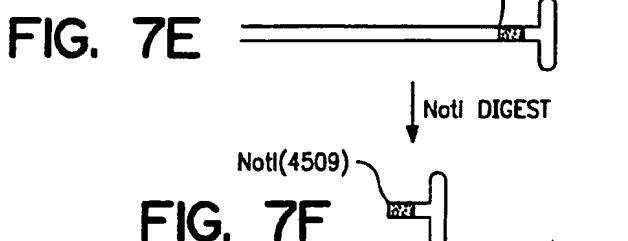
**FIG. 7A****FIG. 7B****FIG. 7C****FIG. 7D****FIG. 7E****FIG. 7F**

FIG. 8A

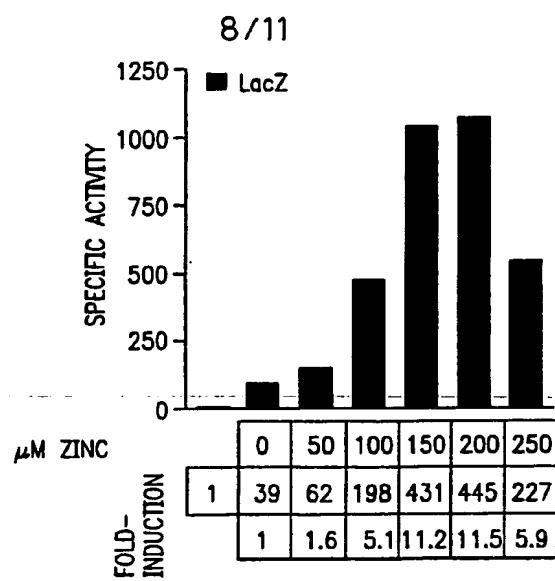


FIG. 8B

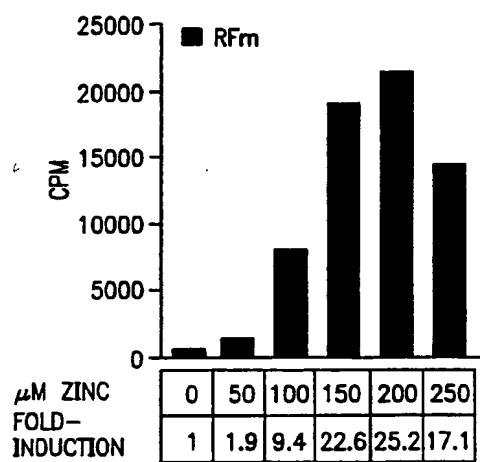
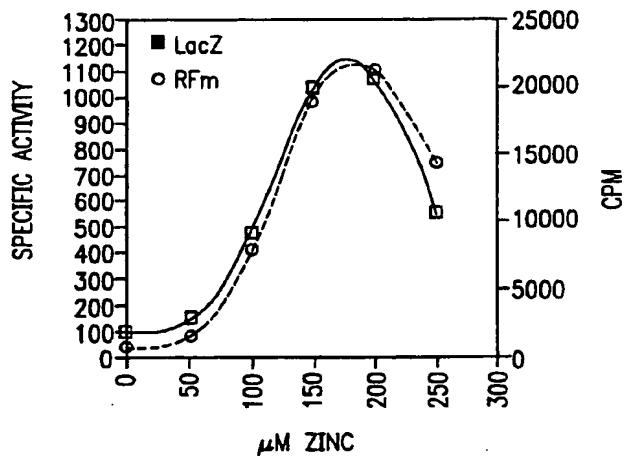


FIG. 8C



9 / 11

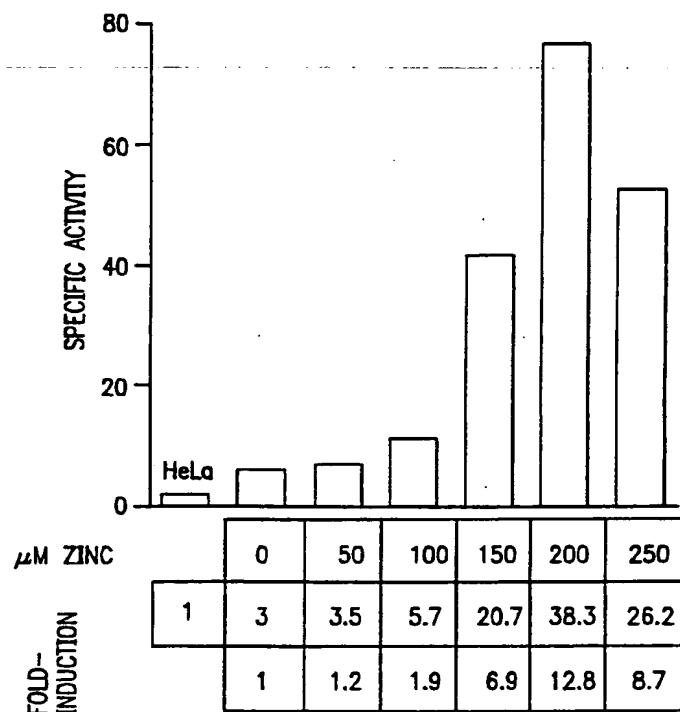


FIG. 9

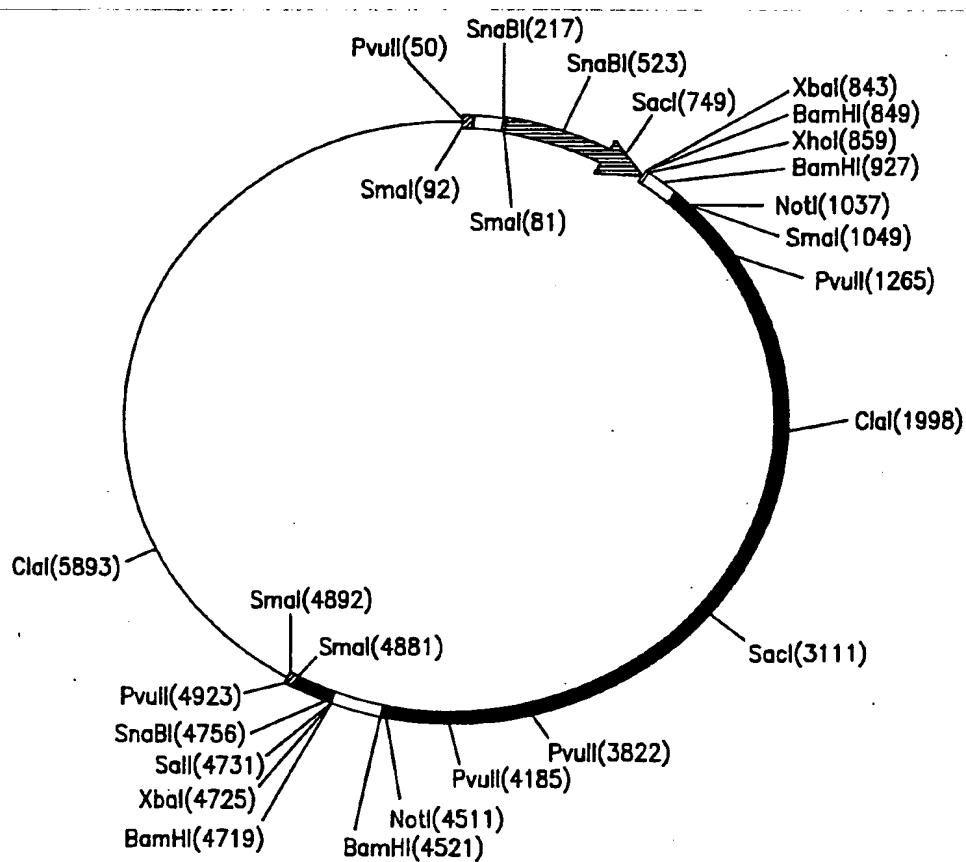


FIG. 10

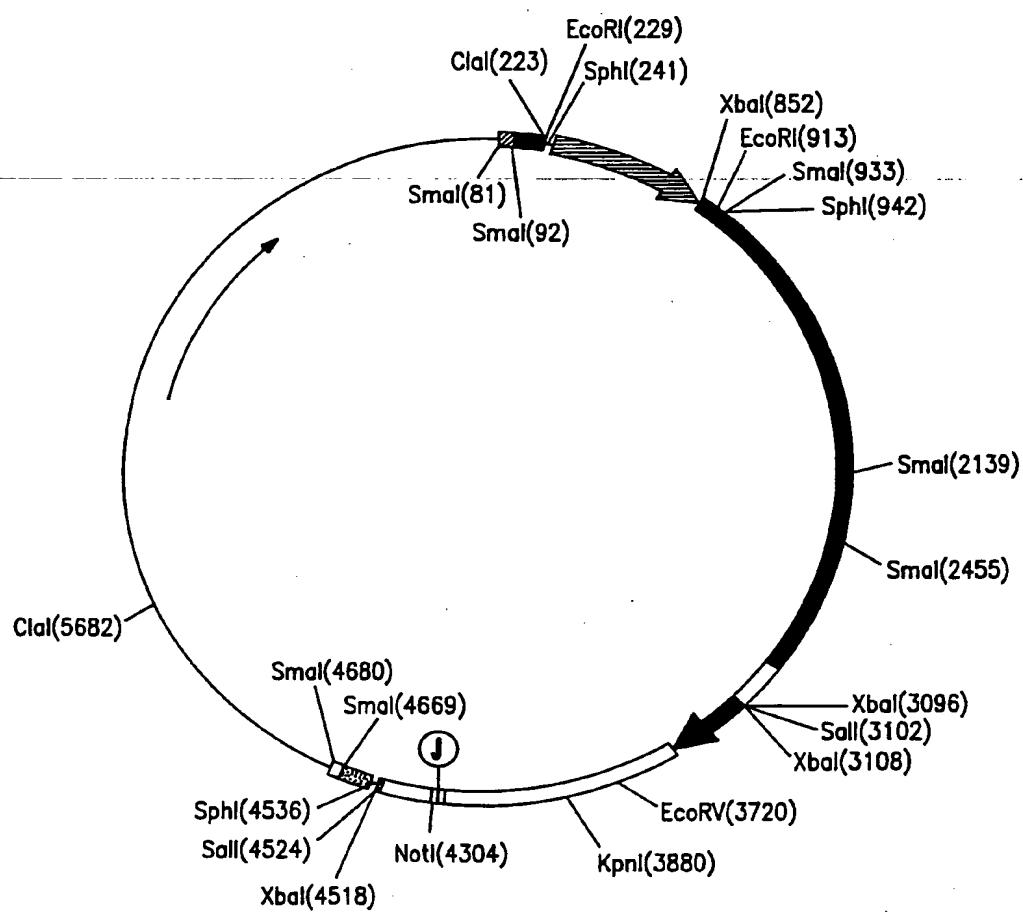


FIG. 11

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/86, 15/87, 5/10, A61K 35/76, 48/00</b>		A3	(11) International Publication Number: <b>WO 96/39530</b> (43) International Publication Date: <b>12 December 1996 (12.12.96)</b>
(21) International Application Number: <b>PCT/US96/10245</b>		Robindale Apartment F5, 1925 Lawrence Road, Havertown, PA 19083 (US).	
(22) International Filing Date: <b>4 June 1996 (04.06.96)</b>		(74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).	
(30) Priority Data: <b>08/462,014 5 June 1995 (05.06.95) 08/549,489 27 October 1995 (27.10.95)</b>		US	(81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(60) Parent Applications or Grants (63) Related by Continuation		Published With international search report.	
US Filed on US Filed on		08/462,014 (CIP) 5 June 1995 (05.06.95) 08/549,489 (CIP) 27 October 1995 (27.10.95)	(71) Applicant (for all designated States except US): <b>THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; 133 South 36th Street, Philadelphia, PA 19104-3246 (US).</b>
(72) Inventors; and (75) Inventors/Applicants (for US only): <b>WILSON, James, M. [US/US]; 1350 N. Avignon Drive, Gladwyne, PA 19035 (US). FISHER, Krishna, J. [US/US]; 4006 Pine Street, Philadelphia, PA 19104 (US). GAO, Guang-Ping [CN/US];</b>		(88) Date of publication of the international search report: <b>5 June 1997 (05.06.97)</b>	
(54) Title: <b>RECOMBINANT ADENOVIRUS AND ADENO-ASSOCIATED VIRUS, CELL LINES, AND METHODS OF PRODUCTION AND USE THEREOF</b>			
(57) Abstract			
<p>An adenovirus E1/E4 expressing packaging cell line is provided, which permits the generation of recombinant adenoviruses deleted in both gene regions. A method for enhancing the efficiency of transduction of a recombinant AAV into a target cell is provided by infecting a target cell with a recombinant AAV comprising a selected transgene under the control of regulatory sequences. The infected cell is contacted with an agent which facilitates the conversion of single stranded recombinant virus to its double stranded form.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

# INTERNATIONAL SEARCH REPORT

Int. onal Application No  
PCT/US 96/10245

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12N15/86 C12N15/87 C12N5/10 A61K35/76 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	J. VIROLOGY, vol. 70, no. 1, January 1996, pages 520-532, XP000570468 FISHER, K.J. ET AL.: "Transduction with recombinant Adeno-Associated Virus for gene therapy is limited by leading-strand synthesis" see the whole document ---	1-7
P, Y		8-23
P, X	J. VIROLOGY, vol. 70, no. 3, March 1996, pages 1845-1854, XP002027273 WEITZMAN, M.D. ET AL.: "Recruitment of wild-type and recombinant Adeno-Associated Virus into Adenovirus replication centers" see the whole document ---	1-7
P, Y		8-23
		-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

1

Date of the actual completion of the international search

10 March 1997

Date of mailing of the international search report

25. 03. 97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentzaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Donath, C

# INTERNATIONAL SEARCH REPORT

Int. Application No.  
PCT/US 96/10245

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 95 20671 A (RHONE-POULENC RORER S.A.) 3 August 1995 see page 2, line 33 - page 3, line 30 see page 5, line 28 - page 7, line 24; examples 2-5 ---	1-7
P,X	WO 96 13598 A (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 9 May 1996 see page 4, line 23 - page 5, line 25 see page 8, line 1 - page 15, line 13 see page 18, line 28 - page 19, line 13 ---	1-7
E	WO 96 22378 A (RHONE-POULENC RORER S.A.) 25 July 1996 see page 3, line 3 - line 21 see page 7, line 17 - line 32 see page 13, line 27 - page 16, line 26 ---	1-7
X	GENE, vol. 119, no. 2, 1992, pages 265-272, XP002027274 NAHREINI, P. ET AL.: "Cloning and integration of DNA fragments in human cells via the inverted terminal repeats of the adeno-associated virus 2 genome" see the whole document ----	1-7
Y	CRC HANDBOOK OF PARVOVIRUSES, ED. P. TIJSSER, vol. I, 1990, pages 155-168, XP002027275 CARTER, B.J.: "The growth cycle of adeno-associated virus" see page 158, last paragraph - page 161, paragraph 1 -----	8-23

1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/10245

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9520671 A	03-08-95	FR 2716682 A AU 1539595 A CA 2181602 A EP 0741793 A FI 962990 A NO 962950 A ZA 9500628 A	01-09-95 15-08-95 03-08-95 13-11-96 26-07-96 12-07-96 23-10-95
WO 9613598 A	09-05-96	AU 4405596 A	23-05-96
WO 9622378 A	25-07-96	FR 2729674 A AU 4544396 A	26-07-96 07-08-96

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADING TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER: \_\_\_\_\_**

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**